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OBESITY, SIRTUINS, CARDIORESPIRATORY FITNESS, AND METABOLIC HEALTH - MODELING ACQUIRED ENVIRONMENTAL EFFECTS

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ACADEMIC DISSERTATION

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"If I have seen further it is by standing on the shoulders of giants."

ABSTRACT

Rates of obesity have risen noticeably during the past few decades in industrialized societies along with a concomitant increase in metabolic diseases such as dyslipidemia, type 2 diabetes, and coronary artery disease. These metabolic diseases contribute significantly to morbidity and mortality, making the study of their mechanisms vital. However, there are many difficulties in investigating the effects of obesity on metabolic health. Cross-sectional studies are likely confounded by genetic factors since obesity and metabolic diseases are highly heritable traits and common genetic factors could explain the observed covariation. It is possible to exclude confounding genetic and early environmental factors by studying samples of twins, which was the methodological focus of Studies I and III. The confounding genetic factors can also be excluded by observing individuals at multiple time-points in a longitudinal interventional study. The focus of Study II was to examine how adipose tissue function responds to a one-year weight-loss intervention in unrelated individuals.

Subcutaneous adipose tissue (SAT) dysfunction has been theorized to drive some of the effects of obesity on metabolic health. Sirtuin (SIRT) proteins and their activity have recently been proposed as possible mediators of subcutaneous adipose tissue dysfunction in obesity. SIRTs are NAD^+-dependent deacylases that regulate various cellular functions (e.g. transcription factors) according to cellular energy levels, as reflected in the availability of intracellular NAD^+. There are numerous animal studies manipulating SIRT and NAD^+ biology, which together seem to show that increasing SIRT activity promotes metabolic health (e.g. insulin sensitivity), whereas reducing SIRT activity has the opposite effect. Thus, the focus of Study I, comprising a sample of healthy Finnish genetically identical monozygotic twins (MZ), was to determine whether obesity is associated with SAT SIRT and NAD^+ synthesis gene expression and how SAT SIRT expression is associated with measures of SAT inflammation and systemic insulin resistance. The genetically informative sample of MZ twins was used to exclude possible confounding by genetic and early environmental factors in these associations. The purpose of Study II, a Finnish longitudinal weight loss intervention study on unrelated individuals, was to further examine how SAT SIRT and NAD^+ synthesis gene expression respond to weight loss. Our results from these studies suggest that SAT expression of SIRT and NAD^+ synthesis genes is reduced by long-term acquired obesity and increased during weight loss, effects that are not confounded by genetic or early environmental factors.

Obesity itself might not be the factor driving metabolic disease, although several studies show associations between obesity and metabolic dysregulation. Poor cardiorespiratory fitness (CRF) has been suggested as an alternative explanation to the metabolic dysregulation observed in obese

individuals. Moreover, differences in CRF between obese individuals has been hypothesized to explain why not all obese individuals exhibit metabolic dysregulation. Some authors have even made the assertion that if CRF is taken into account the association between obesity and increased mortality disappears. The focus of Study III, an analysis of one Finnish and one Danish twin sample, was to determine whether obesity or CRF best explains poor metabolic health. After controlling for confounding by genetic and early shared environmental factors, the results suggest that obesity is strongly associated with the studied metabolic health variables (e.g. insulin sensitivity, metabolic syndrome traits), whereas CRF, defined through maximal oxygen uptake, is only very weakly or not at all associated with metabolic health.

TIIVISTELMÄ

Lihavuus on yleistynyt merkittävästi teollistuneissa yhteiskunnissa viime vuosikymmeninä, minkä myötä metaboliset sairaudet, kuten dyslipidemia, tyypin 2 diabetes ja sepelvaltimotauti ovat yleistyneet. Nämä metaboliset sairaudet johtavat merkittävään sairastavuuteen ja kuolleisuuteen, minkä takia metabolisten sairauksien mekanismien tunteminen on tärkeää. Tutkimus lihavuuden vaikutuksista metaboliseen terveyteen on kuitenkin hankalaa. Poikkileikkaustutkimuksissa geneettiset tekijät toimivat mahdollisina sekoittavina tekijöinä, sillä lihavuus ja metaboliset häiriöt ovat suuressa määrin periytyviä ominaisuuksia. Perimän vaikutukset voidaan kuitenkin vakioida tutkimalla kaksosia, mikä oli Tutkimusten I ja III menetelmällinen tavoite. Perimän vaikutuksia voidaan myös poissulkea pitkittäistutkimuksen keinoin, havainnoimalla samaa yksilöä useassa eri aikapisteessä. Tutkimuksen II menetelmällisenä tarkoituksena olikin arvioida kuinka rasvakudoksen toiminta reagoi vuoden pituiseen laihdutusinterventioon.

Ihonalaisen rasvakudoksen toiminnan häiriöiden on ehdotettu ainakin osittain välittävän lihavuuden vaikutuksia metaboliseen terveyteen. Eräs kirjallisuudessa esitetty rasvakudoksen toiminnan häiriöitä selittävä mekanismi on sirtuiiniproteiinien ekspression ja aktiivisuuden mahdollinen väheneminen lihavuuden seurauksena. Sirtuiinit ovat NAD^+-riippuvaisia entsyymeitä, jotka säätelevät useita eri solujen toimintoja solujen NAD^+ pitoisuuden mukaan. Koska NAD^+-pitoisuudet kuvastavat solujen energiatasoja, sirtuiinit toimivat tavallaan solujen metabolisen tilan sensoreina ja ohjaavat geeniekspressiota sekä entsyymien aktiivisuutta energiatasojen mukaan. Lukuisat koe-eläintutkimukset, joissa manipuloidaan sirtuiini- ja NAD^+-biologiaa, näyttävät, että sirtuiiniaktiivisuuden lisääminen vaikuttaa suotuisasti metaboliseen terveyteen (esim. insuliiniherkkyyteen), siinä missä sirtuiiniaktiivisuuden vähentämisellä on vastakkaisia vaikuksia. Tutkimuksessa I tarkasteltiin suomalaista identtisistä (yksimunaisista) kaksosista koostuvaa otosta ja tarkoituksena oli arvioida, assosioituuko lihavuus ihonalaisen rasvakudoksen NAD^+:n synteesiin ja kulutukseen liittyvien geenien ja sirtuiinigeenien ekspressioon. Lisäksi tarkasteltiin sitä, assosioituvatko sirtuiinigeenien ekspressiotasot ihonalaiskudoksen inflammaatiogeenien ekspressioon ja systeemiseen insuliiniresistenssiin. Kaksosten geneettistä identtisyyttä hyödyntämällä vakioitiin näistä assosiaatioista perimän mahdollinen sekoittava vaikutus pois. Tutkimuksessa II tarkasteltiin laihdutusinterventioon osallistuvia suomalaisia lihavia henkilöitä, jotka eivät olleet kaksosia. Tutkimuksen II tarkoituksena oli tarkastella kuinka ihonalaisen rasvakudoksen NAD^+-synteesiin liittyvien geenien ja sirtuiinigeenien ekspressio muuttuu laihduttamisen seurauksena. Tutkimusten I ja II tulokset yhdessä viittaavat siihen, että ihonalaisen

rasvakudoksen NAD^+-synteesiin liittyvien geenien ja sirtuiinigeenien ekspressio vähenee lihavuuden ja lisääntyy laihduttamisen seurauksena. Näissä tuloksissa on perimän ja aikaisen ympäristön mahdolliset sekoittavat vaikutukset suljettu pois tarkastelemalla kaksosia poikkileikkauksena ja yksilöitä pitkittäisesti.

Lihavuus ei välttämättä itsessään kuitenkaan aiheuta metabolisia häiriöitä, vaikka lukuisissa tutkimuksissa niiden on esitetty liittyvän toisiinsa. Vaihtoehtoiseksi selitykseksi metabolisten häiriöiden kehittymiselle on tarjottu huonoa fyysistä suorituskykyä. Lisäksi, hyvän fyysisen suorituskyvyn on ehdotettu mahdollisesti selittävän miksi jotkut lihavat henkilöt ovat metabolisesti terveitä. On jopa väitetty, että kun fyysisen suorituskyvyn vaikutukset huomioidaan, lihavuus ei ole yhteydessä lisääntyneeseen kuolleisuuteen. Tutkimuksessa III tarkasteltiin yhtä suomalaista ja yhtä tanskalaista kaksosotosta ja tarkoituksena oli vertailla, selittääkö lihavuus vai fyysinen suorituskyky paremmin metabolisen terveyden mittarien vaihtelua. Lisäksi tarkoituksena oli vakioida perimän sekoittavat vaikutukset hyödyntämällä kaksosaineistoja. Tutkimuksen III tulokset viittaavat siihen, että lihavuus assosioituu voimakkaasti tutkittuihin metabolisen terveyden mittareihin (esim. insuliiniherkkyyteen, veren lipideihin ja ektooppisen rasvan määrään). Toisaalta fyysisen suorituskyvyn, jota mitattiin maksimaalisen hapenottokyvyn avulla, huomattiin assosioituvan hyvin heikosti tutkittuihin metabolisen terveyden mittareihin.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I. **Jukarainen S**, Heinonen S, Rämö JT, Rinnankoski-Tuikka R, Rappou E, Tummers M, Muniandy M, Hakkarainen A, Lundbom J, Lundbom N, Kaprio J, Rissanen A, Pirinen E, Pietiläinen KH. "Obesity Is Associated With Low NAD(+)/SIRT Pathway Expression in Adipose Tissue of BMI-Discordant Monozygotic Twins." *The Journal of Clinical Endocrinology and Metabolism*, 2016;101(1):275–283.

II. Rappou E*, **Jukarainen S***, Rinnankoski-Tuikka R, Kaye S, Heinonen S, Hakkarainen A, Lundbom J, Lundbom N, Saunavaara V, Rissanen A, Virtanen KA, Pirinen E, Pietiläinen KH. "Weight Loss Is Associated With Increased NAD+/SIRT1 Expression But Reduced PARP Activity in White Adipose Tissue." *The Journal of Clinical Endocrinology and Metabolism*, 2016;101(3):1263–1273. *The authors contributed equally to the study.

III. **Jukarainen S**, Holst R, Dalgård C, Piirilä P, Lundbom J, Hakkarainen A, Lundbom N, Rissanen A, Kaprio J, Ohm Kyvik K, Sørensen TIA, Pietiläinen KH. "Cardiorespiratory fitness and adiposity as determinants of metabolic health – pooled analysis of two twin cohorts." *The Journal of Clinical Endocrinology and Metabolism*, 2017;101(1):275–283.

The publications are referred to in the text by their roman numerals.

ABBREVIATIONS

BAT	brown adipose tissue
BIGTT-AIR	BIGTT acute insulin response index
BIGTT-SI	BIGTT insulin sensitivity index
BMI	body mass index
BMI SDS	body mass index standard deviation score
BP	blood pressure
Ca-vO_{2max}	maximal arteriovenous oxygen difference
CRF	cardiorespiratory fitness
DZ	dizygotic
FFM	fat-free mass
FFMI	fat-free mass index
FMI	fat mass index
GO	gene ontology
HbA1c	glycated hemoglobin
HDL	high-density lipoprotein cholesterol
HOMA-IR	homeostatic model assessment insulin resistance index
HR_{max}	maximal heart rate
hS-CRP	high-sensitivity CRP
LDL	low-density lipoprotein cholesterol
MZ	monozygotic
NAD^+	nicotinamide dinucleotide
NAMPT	nicotinamide phosphoribosyltransferase
NMNAT	nicotinamide mononucleotide adenylyltransferase
NRK1	nicotinamide riboside kinase
OGTT	oral glucose tolerance test
PARP	poly ADP ribose polymerase protein
PET	positron emission tomography
Q_{max}	maximal cardiac output
RT-PCR	reverse transcription polymerase chain reaction
SAT	subcutaneous adipose tissue
SIRT	sirtuin
STAC	SIRT1-activating compound
SV_{max}	maximal stroke volume
T2DM	type 2 diabetes mellitus
UPR^{mt}	mitochondrial unfolded protein response
VAT	visceral adipose tissue
VO_{2max}	maximal oxygen uptake/consumption [ml/min]
VO_{2max}/FFM	VO_{2max} divided by fat free mass [ml/(min×kg)]

1 INTRODUCTION

Obesity is associated with metabolic syndrome, type 2 diabetes mellitus (T2DM), hypertension, dyslipidemia, and coronary artery disease[1], which together contribute significantly to morbidity and mortality globally. The development of these diseases is a result of dysregulated metabolism, where insulin resistance[2,3], subcutaneous adipose tissue (SAT) dysfunction[4], and ectopic fat accumulation[5,6] possibly play central roles. The environmental factors contributing to the pathogenesis of these obesity-related disease states have been topics of active research. For example, dietary factors, such as excess fat[7] or sugar[7–10] intake, have been implicated in contributing to the development of these obesity-related comorbidities. It must be noted though that the relationship between obesity and development of metabolic disease is not perfect, and not all obese individuals develop metabolic disturbances[11–13]. Additionally, physical activity and cardiorespiratory fitness (CRF) have been proposed as factors that help prevent or reverse the development of metabolic disease[12–15], possibly explaining why some obese individuals are metabolically healthy.

The mechanisms through which obesity causes metabolic dysfunction remain somewhat obscure. Some theories link the combination of caloric excess and a sedentary lifestyle to the development of SAT dysfunction, which might cause metabolic dysregulation through altered secretion of adipokines (signal molecules secreted by the adipose tissue)[4,16], increased SAT and consequent systemic inflammation[5,17,18], and lipid overflow into other tissues[2,5,6] (due to inadequate adipogenesis and SAT insulin resistance). The exact mechanisms behind the altered physiology of SAT in obesity are not well known, but the reduced activity of the NAD^+/SIRT1 pathway[19–21] and the related mitochondrial dysfunction observed in obesity[22–25] have recently been proposed as possible mechanisms linking unhealthy lifestyle factors with SAT dysfunction. Sirtuin proteins (SIRTs) regulate various cellular functions through interacting with histone proteins, enzymes, and transcription factors according to cellular energy levels reflected in cellular nicotinamide dinucleotide (NAD^+) availability[19]. NAD^+ is a cosubstrate consumed by sirtuin proteins in their enzymatic deacylase activity, and thus, the activity of SIRTs is linked to cellular energy levels, insofar as SIRT activity reflects the availability of cellular NAD^+[19,26]. Along with sirtuins, Poly ADP ribose polymerase proteins (PARPs) are important consumers of cellular NAD^+[23,26], competing with sirtuin proteins for the same cellular NAD^+ pools. The purpose of Studies I and II in this thesis was to investigate the effects of acquired obesity in monozygotic (MZ) twins (Study I) or weight loss (Study II) on SAT NAD^+ biosynthetic enzyme expression, SAT PARP activity or expression, and SAT *SIRT* expression. All of these are potentially involved in the pathogenesis

of metabolic dysfunction related to obesity in humans through their effects on SAT function.

Cardiorespiratory fitness (CRF) has been suggested to be an important factor in preventing or even reversing metabolic dysfunction in metabolic disease[12–14]. Furthermore, a recent meta-analysis by Barry et al.[15] purports to show that if you take CRF into account, obesity does not independently contribute to all-cause mortality. The purpose of Study III was to compare adiposity and CRF as predictors of various measures of metabolic health in two Nordic cross-sectional samples of MZ and DZ (dizygotic) twins. While Studies I and III are twin studies and Study II is a longitudinal weight loss intervention study in unrelated individuals, all three studies model the unique environmental associations between variables, controlling for genetic and early environmental factors, either by observing the same set of individuals across time (Study II) or controlling for genetic and shared early environmental factors by examining genetically identical MZ twins (Studies I and III).

2 REVIEW OF THE LITERATURE

2.1 TWIN STUDIES

There are multiple possible interventions that could improve metabolic health in the context of obesity and sedentary lifestyle. Thus, studying the environmental factors affecting metabolic health is important. While evaluation of the heritability or genetics of a disease state, such as type 2 diabetes mellitus (T2DM), offers a way to better understand the pathogenesis of the disease, knowledge of environmental factors affecting the disease is more closely related to designing interventions to address the problem of acquired metabolic disease. If a traditional cross-sectional study on unrelated individuals finds an association between the two variables X and Y, the covariation between X and Y could in theory come from three different sources: 1) there could be common genetic factors causing both X and Y (a confounded genetic association), 2) X could cause Y (a causal environmental association), and 3) there could be a shared environmental factor Z (or factors) causing both X and Y (a confounded environmental association) (Figure 1).

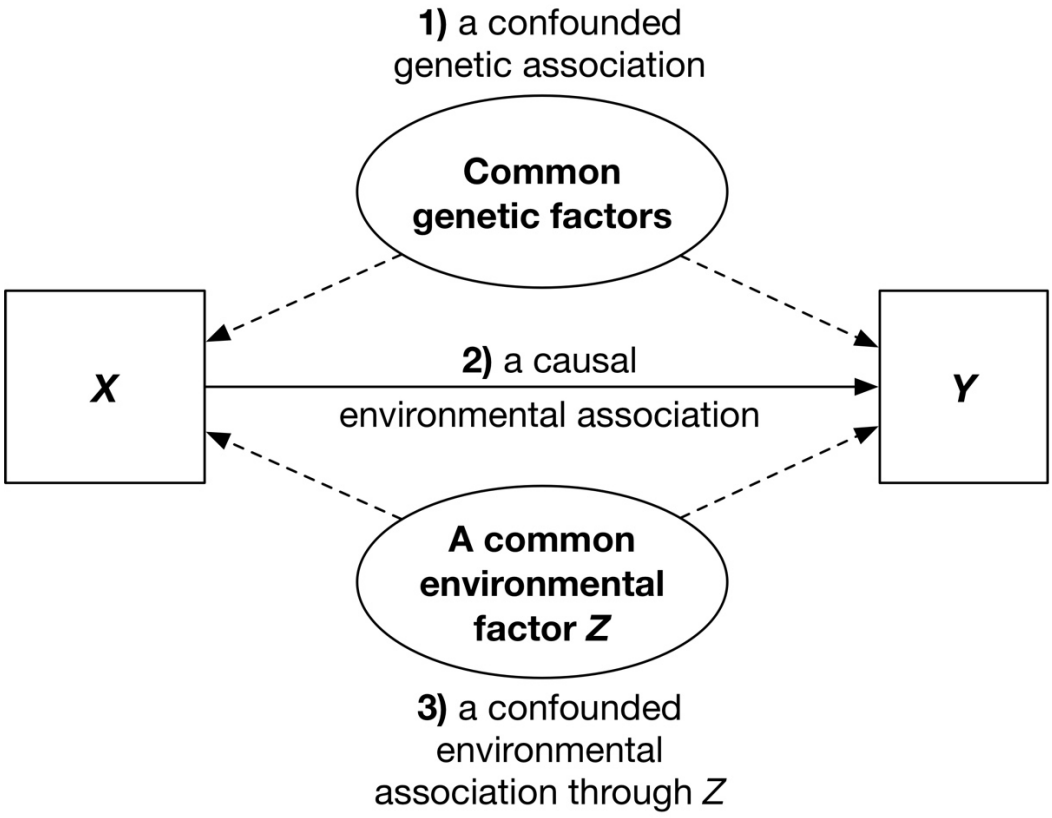

Figure 1 Possible sources of covariation between the two variables X and Y.

While discovering associations from source 2 (causal environmental associations) is the most common goal in research, cross-sectional studies do not really provide information specifying whether source 1, 2, or 3, or any combination of these, underlies an observed association. A common way to investigate whether there truly is a causal association between two variables is to perform a longitudinal intervention study, such as a randomized controlled trial, where X can be changed and then Y is observed to see whether a change follows. Since each individual is observed at multiple time points, the genetic and past environmental factors are held constant, thus precluding confounding effects from sources 1 and 3. Ideally, because of randomization, every factor except the manipulation of X is randomly distributed among the intervention group and the control group. Therefore, any nonrandom changes in Y must be due to changes in X. While randomized controlled trials are in some ways ideal studies for figuring out whether environmental interventions have certain effects, they are not always feasible or possible, especially when studying effects that unfold over a long period or when the intervention is costly, unfeasible, or unethical to perform.

Because in twin samples the degree to which related individuals share segregating genes on average is known (for DZ twin pairs 50%, for MZ twins 100%), it is possible to estimate the extent to which the variances in traits X and Y are due to genetic or environmental influences[27]. Also it is possible to estimate the extent to which covariation in X and Y is due to shared genetic or environmental influences and to determine the genetic and environmental correlations between the variables[27]. A causal association between X and Y (source 2) and a shared environmental factor causing both X and Y (source 3) can account for an environmental correlation between X and Y, whereas a common genetic cause of X and Y is not present in an environmental correlation. While it is not possible in a cross-sectional twin study to determine whether covariation between X and Y is causal (source 2) or due to common confounding environmental factors (source 3), it is possible to exclude genetic confounding (source 1) from an association between two variables. It is also possible to exclude confounding from shared environmental factors common to the twin pairs, such as familial and maternal factors (a part of source 3).

In summary, to exclude the possibility of genetic confounding, at least two possibilities arise: to examine the same unrelated individuals longitudinally at multiple time points (Study II) or to examine a genetically informative sample of twins (or families) cross-sectionally (Studies I and III).

2.1.1 BIOLOGY OF TWINS

Twin pairs can be classified into three kinds: monozygotic twins, same sex dizygotic twins, and opposite sex dizygotic twins. DZ twin pairs develop from two different eggs fertilized by two different spermatozoa and are essentially akin to two siblings developing in the same womb, thus sharing 50% of their

segregating genes. DZ twins develop within different amnions and have their own separate chorions. MZ twin pairs, however, arise from the same egg fertilized by the same spermatozoon, thus sharing 100% of their segregating genes (not taking post-zygotic somatic mutations into account). MZ twins most commonly share the same placenta, but have different amnions (70-75% of all born MZ twin pairs), but they can also have separate placentae and amnions (25-30% of all born MZ twin pairs) as DZ twins do, and rarely they can share both the same placenta and the same amnion (1-2% of all born MZ twin pairs). MZ twin pairs with different placentae and amnions are thought to be separated from days 0 to 3 after fertilization, MZ pairs with a shared placenta but different amnions are thought to be separated between days 4 and 7 after fertilization, whereas MZ pairs with a shared placenta and amnion are thought to be separated between days 7 and 14 after fertilization[28].

Due to the special nature of twin pregnancies, twins differ somewhat from singletons from normal pregnancies with respect to their early uterine environment and gestation period. While the average singleton pregnancies last for 39 weeks, twins are born earlier, at 35 weeks on average[29]. Twin pregnancies (especially MZ pregnancies with shared placentae) also predispose the fetuses to increased complications such as twin-to-twin transfusion syndrome[29]. Twin pregnancies are also associated with more pronounced changes in maternal physiology and maternal complications[29]. Despite MZ twins being virtually genetically identical, differences in the uterine environment result in around 10% of born MZ twins to be apparently discordant at birth[28]. The fact that twin pregnancies, especially MZ twin pregnancies, differ from singleton pregnancies has some possible implications for generalizing results from twin populations to singleton populations.

2.1.2 HERITABILITY ANALYSIS

Twin samples provide an opportunity to investigate the sources of variation in traits by decomposing observed variation into genetic and environmental sources. Because MZ twins share 100% of their segregating genes and DZ twins share on average 50% of their segregating genes, it is possible to investigate the contributions of latent genetic and environmental factors to different phenotypic traits with a sample of MZ and DZ twins. Phenotypic variation of traits in twin or family models is usually modeled to follow from four latent variables: additive genetic variance (A), shared environmental variance (C), unique environmental variance (E), and dominance variance (D)[27]. The full model (ACED) model then contains all of the four latent variables. With a dataset of MZ and DZ twins, it is only possible to solve for three of these latent variables, and often dominance variance is left out of the model (ACE model, Figure 2)[30].

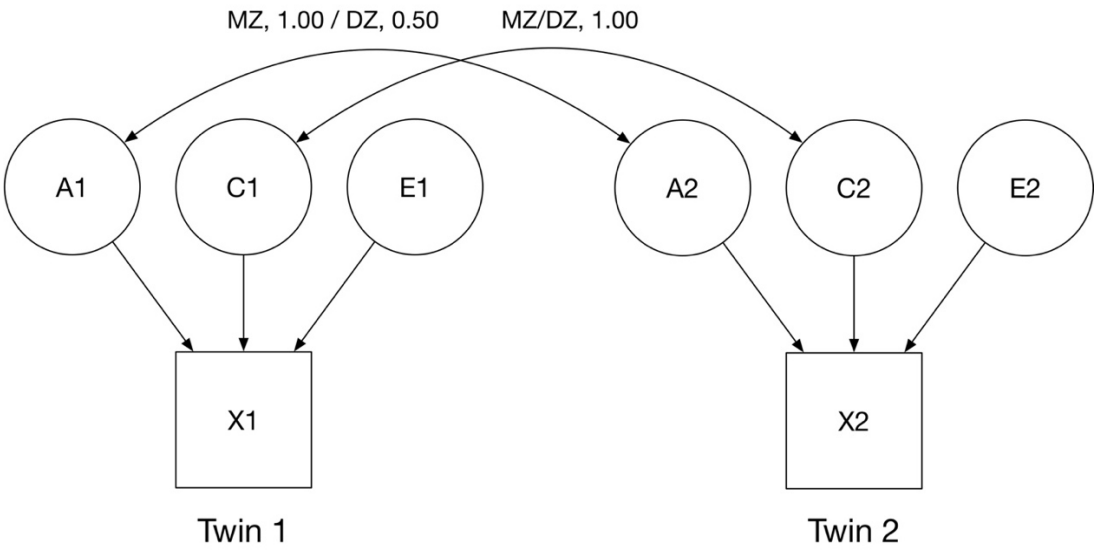

Figure 2 A simplified variance components model of a single trait univariate ACE model for MZ/DZ twin pairs for trait X. A, C, and E stand for the latent additive genetic, shared environmental, and unique environmental variance variables for twins 1 and 2 that cause the observed variables X1 and X2 for each twin. The latent additive genetic variance (A) has a covariance of 1.00 in MZ pairs and 0.50 in DZ pairs due to their respective degrees of genetic relatedness. The latent shared environmental variance (C) has a covariance of 1.00 since, by definition, the environment is shared by twin pairs reared together. The latent unique environmental variance (E) has a covariance of 0 in MZ and DZ pairs by definition. Adapted from Neale & Maes[27].

One common application of a genetically informative sample of MZ and DZ twins is the estimation of the heritability of a trait. While the focus of my research in this dissertation was not in estimating heritabilities of traits per se, understanding heritability is still relevant. Broad sense heritability (H^2) is defined as:

$$H^2 = \frac{Var_G(X)}{Var_P(X)} \quad ,$$

where $Var_P(X)$ stands for the total phenotypic variance of trait X and $Var_G(X)$ stands for the combined genetic contributions (e.g. additive genetic variance, dominance variance, epistatic or multigenic interactions, maternal and paternal effects)[27]. So, heritability is the proportion (from 0 to 1) of all variation in a trait in the population explained by genetic variability in the population. However with a sample of MZ and DZ twins reared together, it is only possible to solve for three of the ACED latent variables, with an ACE decomposition of variance being commonly of interest, since variance accounted for by dominance effects is generally considered small[27,31]. The proportion of variance explained by the latent A variable to total phenotypic variance is called narrow sense heritability (h^2 or a^2):

$$h^2 = a^2 = \frac{Var_A(X)}{Var_P(X)} \quad ,$$

where $Var_A(X)$ is the additive genetic variance in trait X. While narrow sense heritability is easier to estimate than broad sense heritability, it gives slightly lower estimates for heritability. Similarly, the percentage of variation attributable to shared environmental (c^2) and unique environmental (e^2) factors from an ACE model can be expressed as:

$$c^2 = \frac{Var_C(X)}{Var_P(X)} \qquad e^2 = \frac{Var_E(X)}{Var_P(X)} \quad ,$$

where $Var_C(X)$ is the shared environmental variance in X and $Var_E(X)$ is the unique environmental variance in X. Heritability is of interest in health research because if traits are highly heritable, environmental effects behind variability in that trait in the population are small and vice versa. Understanding which factors are behind variation in a trait might help when thinking about the kinds of interventions that could be effective in improving health outcomes.

2.1.3 MONOZYGOTIC TWIN MODELS

When trying to understand the causal associations between variables (whether *X* causes *Y* for example), a classical approach is to model the association in a cross-sectional sample by fitting a statistical model on the individual values of the variables (e.g. the Pearson correlation of *X* and *Y*). One major problem with these kinds of studies is that they cannot determine whether the covariation between the traits is due to environmental or genetic factors (Figure 1). Common genetic factors behind both traits *X* and *Y* could be account for the covariation, thus confounding the purported causal association between *X* and *Y*.

In research on obesity, fitness, and metabolic health, the environmental pathway of covariation between variables is of special interest because the effects of lifestyle factors (e.g. diet and physical activity) and interventions go through the environmental pathway (although they may be moderated by genetic effects). One way to estimate the environmental covariation between traits is by examining the correlation of intrapair differences of the variables in a sample of MZ twins[32,33]. I define intrapair difference of a variable X (ΔX) as $\Delta X = X_{twin2} - X_{twin1}$. Because MZ twins in practice share 100% of their genes and, by definition, the shared environmental effects (C) are shared by co-twins, any differences between co-twins within a MZ twin pair must be due to unique environmental effects (E). Following that, the correlation between ΔX and ΔY within MZ twin pairs ($r_{MZ}(\Delta X, \Delta Y)$) depicts the unique environmental (E) covariance between traits X and Y that is not confounded by genetic (A) or shared environmental (C) factors. In MZ intrapair regression models for Study III, the standardized regression coefficients (βs), analoguous to correlation coefficients, are interpreted similarly to $r_{MZ}(\Delta X, \Delta Y)$. Because genetic and shared environmental factors are excluded from a correlation

between ΔX and ΔY in MZ twin pairs, $r_{MZ}(\Delta X, \Delta Y)$ is stronger evidence for a causal association between those variables than a mere phenotypic correlation in unrelated individual subjects[33].

2.1.4 BIVARIATE GENETIC MODELS USING MZ AND DZ TWINS

The classical univariate twin model (Figure 2) can be extended to include more than one variable, so that, in addition to partitioning the variance of variables into ACE components, the shared ACE variances and covariances of variables are also modeled. This allows the calculation of genetic and environmental correlations between two variables through additive genetic (A), shared environmental effects (C), and unique environmental effects (E). The models used in this setting are commonly referred to as *Cholesky decomposition models*[27].

Estimated variances and covariances from a bivariate Cholesky decomposition model can be used to calculate additive genetic (r_A), shared environmental (r_C), and unique environmental (r_E) correlations between two traits:

$$r_A = \frac{Cov_A(X,Y)}{\sqrt{Var_A(X) \times Var_A(Y)}} \quad r_C = \frac{Cov_C(X,Y)}{\sqrt{Var_C(X) \times Var_C(Y)}} \quad r_E = \frac{Cov_E(X,Y)}{\sqrt{Var_E(X) \times Var_E(Y)}} \quad ,$$

where $Cov_A(X,Y)$ is the additive genetic covariance of X and Y, $Var_A(X)$ is the additive genetic variance for X, $Var_C(X)$ is the shared environmental variance of X, and $Var_E(X)$ is the unique environmental variance of X. Note that these correlations depict covariances standardized to the variance of the respective latent variables, not total phenotypic variance. The correlations obtained (r_A, r_C, and r_E) can be expressed as scaled to total phenotypic variance (as r_a, r_c, and r_e) as follows:

$$r_a = \sqrt{h_X^2 \times h_Y^2} \times r_A \qquad r_c = \sqrt{c_X^2 \times c_Y^2} \times r_C \qquad r_e = \sqrt{e_X^2 \times e_Y^2} \times r_E \quad ,$$

where h_x^2 is the narrow sense heritability in trait x, c_x^2 is the proportion of total phenotypic variance attributable to C, and e_x^2 is the proporition of total phenotypic variance attributable to E, as defined earlier.

Following this, r_a, r_c, and r_e are the parts of the phenotypic correlation accounted for by the respective additive genetic, shared environmental, and unique environmental effects, so that $r_p = r_a + r_c + r_e$, where r_p is the phenotypic correlation. r_A, r_C and r_E can be interpreted as the correlation between the respective latent A, C and E variables behind the traits. The distinction between the coefficients standardized to total phenotypic variance (e.g. r_a) and the coefficients standardized to their respective variances (e.g. r_A) is important. For example, even with a very high additive genetic correlation (e.g. $r_A = 0.9$), but with very low heritability of traits X and Y ($h_X^2 = 0.1$ and $h_Y^2 = 0.1$), the contribution of the additive genetic correlation to the total phenotypic correlation might be very small ($r_a = \sqrt{h_X^2 \times h_Y^2} \times r_A = \sqrt{0.1 \times 0.1} \times 0.9 = 0.09$). So, the latent A, C, and E factors behind traits can be

highly correlated, but the extent that this correlation contributes to the phenotypic correlation is determined by the proportions of variance in the two traits accounted for by each latent factor.

Interestingly, the MZ twin intrapair difference correlation $r_{MZ}(\Delta X, \Delta Y)$ between variables X and Y can be thought of as estimating the unique environmental correlation r_E obtained from a bivariate Cholesky decomposition model, since both methods control for shared environmental and genetic influences. This makes a limitation in the MZ twin intrapair differences method (estimating $r_{MZ}(\Delta X, \Delta Y)$) more apparent; since it approximates the covariation in traits due to unique environmental effects (the r_E), while leaving out genetic and shared environmental variance, it does not reveal the proportion of total phenotypic covariance explained by the unique environmental covariation (the r_e). Thus, as in the example in the above paragraph, if the two traits X and Y are not mainly determined by unique environmental influences E, and the e_X^2 and e_Y^2 are low, even a high MZ twin intrapair difference correlation might correspond to a minor part of the phenotypic correlation when scaled to total phenotypic variance (r_e), since $r_e = \sqrt{e_X^2 \times e_Y^2} \times r_E$. Additionally, when calculating intrapair differences, measurement error is compounded, which probably leads to the attenuation of the estimated correlations[33].

In summary, by applying a bivariate ACE Cholesky decomposition model in a sample of MZ and DZ twins one can estimate the A, C, and E variance components in variables X and Y, along with the r_A, r_C, and r_E correlations, and the r_a, r_c, and r_e correlations scaled to total phenotypic variance. However, the unique environmental correlation r_E can be more easily approximated by correlating MZ intrapair differences in variables X and Y: $r_E \sim r_{MZ}(\Delta X, \Delta Y)$. In Studies I and III, we used the $r_{MZ}(\Delta X, \Delta Y)$ method to estimate the unique environmental correlations between variables.

2.1.5 CAUSAL INFERENCE AND TWIN STUDIES

Generally, the problem of causal inference can be approached through a counterfactual (or potential outcomes) framework[33,34]. Following McGue et al.[33], say we are estimating the effects of a dichotomous exposure (or treatment) X on Y, and define $X = T$ if an individual is exposed to X and $X = C$ (or control) if an individual is not exposed to X. The causal effect (e_i) of exposure X on outcome Y in individual i can be expressed as the counterfactual: $e_i = Y_i^T - Y_i^C$, where Y_i^T is the value of Y of individual i with the exposure T, and Y_i^C is respectively the same had there been no exposure. However, since we cannot observe the same individual with an exposure and without, an individual causal effect is only a theoretical quantity that cannot be directly measured. The individual causal effects can however be indirectly estimated if we assume that a population causal effect corresponds to individual causal effects. An average population causal effect for population i

can be defined as: $\bar{e}_i = \bar{Y}i^T - \bar{Y}i^C$, where $\bar{Y}i^T$ and $\bar{Y}i^C$ are the averages of individual effects in populations exposed and not exposed. The gold standard method for estimating these average effects is the randomized controlled trial, where all individuals are randomized to treatment (exposure) or no treatment (no exposure). Because individuals are random with respect to all other factors than the treatment, on average, the treated individuals can serve as counterfactuals to those who were not treated, making the difference between groups in the trial correspond to the average population causal effect, even though individual effects are not directly observed.

Twin studies can be examined from the same framework[33]. Because MZ co-twins are identical in genetic factors and shared environmental factors, we can counterfactually think of different co-twins of a MZ twin pair as a natural experiment demonstrating what would have happened had the "same person" lived in two different unique environments. Making the causal effect of the difference in those co-twins' (twins i and j) exposures: $e_{ij} = Y_i^T - Y_j^C$, where Y_i^T is the value of Y in twin i with unique environmental exposure T (i.e. obese co-twin) and Y_j^C is the value of Y in twin j with unique environmental exposure C (i.e. lean co-twin). Thus, for example, if we examine BMI-discordant MZ twin pairs as in Study I, and observe that heavier co-twins are more insulin resistant compared to their leaner co-twins, we can say that the environmental differences that have led the heavier co-twin to be more obese are associated with the development of higher insulin resistance, and that this association is not due to genetic and environmental factors shared between the co-twins. Therefore, MZ twin pairs can be thought of as natural experiments, where the same "individual" has lived in two different environments. While the abovementioned examples have dichotomous exposures, the same reasoning can be generalized to continuous exposures, such as intrapair BMI difference (modeling the $r_{MZ}(\Delta X, \Delta Y)$). Whereas confounding by genetic factors and shared environmental factors can be excluded in this analysis, it is not possible to determine the exact environmental factors behind the covariation between exposure and outcome, thus the association can be confounded by some unique environmental factors. Neither can reverse causation be excluded. Thus, it could be for example that insulin resistance causes high BMI (reverse causation), or that differences in gut microbiota are both responsible for a high BMI and insulin resistance (confounding).

Along with the MZ intrapair correlation method, a more common way of controlling for genetic confounding in an association between two variables is to perform a longitudinal study and model the changes in the said variables. This is what we did with the weight loss study participants in Study II, who were unrelated individuals and not twins. Although observational longitudinal studies on unrelated individuals and cross-sectional studies on MZ twins have differing study designs, they ultimately answer the same kinds of questions, e.g. what is the environmental association between the studied variables (Table 1). While longitudinal studies with an intervention can show that the intervention X is causally responsible for the effects on Y, observational

longitudinal and cross-sectional studies on MZ twins cannot directly specify which environmental factors are responsible for Y. However, there are some relative advantages in observational longitudinal or cross-sectional twin studies compared with longitudinal interventional studies. They do not require the maintenance of costly and laborious interventions across long timespans, and as observational studies, they are less constrained by study ethics due to the lack of interventions. There are of course observational studies utilizing natural experiments and pseudo-experimental methods, such as mendelian randomization studies[35], that can avoid these problems.

Table 1. *Comparison between longitudinal and MZ intrapair difference study designs.*

	Randomized controlled trial studies	**Observational longitudinal studies**	**MZ intrapair difference studies**
Environment studied	Changes in Y due to short-term environmental intervention on X	Environmental association between changes in X and Y	Long-term environmental association between X and Y
Able to specify the environmental factors behind changes in Y	Maybe (the intervention on X)	No	No
Control for confounding by genetic factors	Yes (same person, different time/environment)	Yes (same person, different time/environment)	Yes (same genetic factors, different environment)
Control for confouding by environmental factors other than X	Yes	No	No

2.2 OBESITY

2.2.1 OVERVIEW OF OBESITY AND METABOLIC HEALTH

Obesity is commonly defined as a BMI of over 30 kg/m^2 whereas a overweight corresponds to a BMI of 25-30 kg/m^2[1]. Obesity or overweight itself is not necessarily a medical problem. Rather, it is the metabolic problems associated with obesity, such as metabolic syndrome characterized by insulin resistance, impaired glucose tolerance, dyslipidemia, and high blood pressure[36], or outright type 2 diabetes mellitus (T2DM), that pose a medical challenge. Metabolic syndrome and T2DM are overlapping conditions, and both are associated with cardiovascular disease. Additionally obesity is also associated with mechanical problems, such as arthritis, musculoskeletal problems, and obstructive sleep apnea, and mental problems, such as depression and anxiety[1], but in this thesis the main focus is on metabolic factors, mainly insulin resistance and dyslipidemia.

BMI is robustly associated with all-cause mortality with a U-shaped curve[37], where the lowest mortality is present at 20-25 kg/m^2 or at 23-24

kg/m² in never smokers[38], with mortality risk rising steadily with increasing BMI. Disease-specific mortality for coronary heart disease, stroke, respiratory disease, and cancer all follow the same pattern[37]. The risk for T2DM depends heavily on obesity or BMI. In the Health Professionals' Study, men with a BMI of at least 35 kg/m² had a 42-fold higher relative risk for T2DM than men with a BMI of less than 23 kg/m², after adjusting for age, family history of diabetes, and smoking[39]. In the Nurses Cohort study, age-adjusted relative risk of T2DM was 28 times higher for women with a BMI around 30 kg/m² and 93 times higher for women with a BMI of at least 35 kg/m², compared with women with a BMI of less than 21 kg/m²[39].

BMI is highly heritable, and as with other traits the heritability varies with age. In a twin study with participants aged 0.5 to 19.5 years[40], the lowest observed heritability of BMI was 41% for girls and 42% for boys at 4 years of age, with the heritability rising to a peak of 75% in 19-year-olds. In one meta-analysis[41], the median heritability of BMI estimated from twin samples containing children, adolescents, and adults was 75%. However, fairly recent family studies of heritability seem to indicate that the estimates for the heritability of BMI from twin studies are overestimates and that the heritability of BMI seems to be 42% in family studies[42,43]. It must be emphasized though that a high heritability estimate of a trait does not imply that it is largely immutable to changes in the environment[44] (e.g. changes in taxation of high-energy/density products). Since a heritability estimate is the ratio of genetic variance in the trait to the total variance in the trait *in a given environment*, it does not say anything about possible variation due to different environmental influences. An example of this is human height, which has a high heritability of around 80%[44], but nevertheless the mean height in Westernized countries has risen considerably since the 19th century[45]. Similarly, phenylketouria is a genetic disorder in which the metabolism of phenylalanine into tyrosine is defective, leading to brain damage and mental retardation if a source of phenylalanine is included in one's diet. Phenylketouria would have had a very high heritability for its clinical phenotype when the treatment for the disease, restricting dietary phenylalanine content, was not known. However, nowadays phenylketouria is recognized and treated early by restricting dietary phenylalanine, and consequently the phenotype of the disease has very low heritability[46]. The same caveats apply to estimates of genetic or environmental correlations; in one environment, an environmental correlation (or even a causal environmental association) could be absent, but in another environment, it could be present.

Similarly, the heritability of cardiorespiratory fitness (CRF), defined as maximal oxygen uptake (VO_{2max}), is also relatively high. In a study of Finnish twins, the heritabilities of VO_{2max} and VO_{2max} divided by fat free mass (FFM) were estimated at 65% and 71%, respectively[47]. In a meta-analysis of several twin studies, the estimated heritabilities were 60% for absolute VO_{2max} and 55% for VO_{2max} divided by weight[48].

T2DM is also highly heritable, with a heritability of around 64-79% in Finnish twins[49,50]. Additionally, in a Finnish family study, T2DM with age at onset between 35 and 60 years had a heritability of 69%, whereas when including patients with onset occurring up to 75 years of age, the heritability estimate was lower, 31%[51], implying that T2DM with earlier onset is more determined by genetic factors than T2DM with late onset. The Discordant Twin (DISCOTWIN) Consortium, incorporating nearly 35 000 twin pairs from seven countries, provides a meta-analyzed heritability estimate of 72% for T2DM across the twin samples[52]. Interestingly, in a Finnish twin study using an AE model, the additive genetic correlation between BMI and T2DM is in men 0.40 and in women 0.45, thus, despite very high heritabilities of both traits, approximately only one-fifth of the covariance between BMI and T2DM is due to common genetic influences[50]. Results from American Indian[53] and Australian[54] populations give similar estimates of the genetic correlation between BMI and T2DM. Interestingly, in the Finnish study, the environmental correlations between BMI and T2DM were even lower at 0.37 in men and 0.22 in women[50], which, along with the high heritability estimates of BMI and T2DM, suggests that only a small amount of phenotypic covariation in BMI and T2DM in Finnish twins is actually due to environmental effects. This does not however mean that an intervention that lowers BMI, such as weight loss due to dieting or increased physical activity, will not effectively prevent T2DM or make the disease less severe since these estimates from twin studies only examine the genetic and environmental factors that have been present in the studied population.

Similarly, insulin sensitivity and insulin resistance, important factors in T2DM, are also moderately heritable. Depending on the study population and the measure, heritability estimates seem to vary from 23% to 60%[55–63]. Furthermore, other components of metabolic syndrome, besides adiposity and insulin resistance, such as blood lipids and blood pressure, are also at least moderately heritable. Depending on the study, the estimates for the heritabilities of plasma LDL (low-density lipoprotein), HDL (high-density lipoprotein), and triglycerides are mostly over 50%[64], and estimates for the heritabilities of systolic and diastolic blood pressure range from around 20% to 70% and from 10% to 50%, respectively[65].

In summary, BMI, VO_{2max}, T2DM, insulin sensitivity, lipids, and blood pressure are moderately or highly heritable traits, emphasizing the need to study them with methods that can control for their genetic background and for possible genetic correlations between the traits when evaluating their associations. This is because a phenotypic association between two variables can be accounted for by a shared genetic background, shared environmental influences, or a causal environmental association between the variables (Figure 1).

2.2.2 PATHOGENESIS OF INSULIN RESISTANCE

Insulin resistance is central to metabolic syndrome, the defining factor of T2DM along with impaired insulin secretion, and involved in the pathogenesis of dyslipidemia[3], and thus, is a central topic in this thesis. Insulin resistance is a phenomenon in which normally insulin-sensitive tissues, such as subcutaneous adipose tissue (SAT), skeletal muscle, and hepatic tissue, do not react to circulating plasma insulin levels in a normal way[3]. Samuel & Shulman[3] provide a succinct review of the current understanding of the pathophysiology of insulin resistance. During a postprandial state, in an insulin-sensitive individual, elevated circulating insulin suppresses hepatic gluconeogenesis and stimulates glycogen synthesis along with *de novo* lipogenesis. Insulin also suppresses adipose tissue lipolysis and the release of free fatty acids into the bloodstream. Finally, insulin stimulates skeletal muscle glucose uptake by translocating GLUT4 receptors into the cell membrane and increases muscle glycogen synthesis[3].

However, in an insulin-resistant individual, during a postprandial state the responses of normally insulin-sensitive tissues to circulating insulin are blunted (Figure 3). Adipose tissue continues lipolysis and release of free fatty acids into the bloodstream. Skeletal muscle does not increase glucose uptake and glycogen synthesis adequately to lower plasma glucose, which leads to impaired glucose tolerance and prolonged elevated postprandial glucose levels. Insulin fails to suppress hepatic gluconeogenesis and to stimulate glycogen synthesis, exacerbating the elevated plasma glucose levels. Increased availability of hepatic glucose due to elevated circulating glucose levels, combined with hepatic lipogenesis not being properly suppressed by insulin due to hepatic insulin resistance, leads to increased hepatic lipogenesis from glucose. The elevated circulating glucose levels also lead to a compensatory increase in insulin release from the pancreatic ß-cells, which presents as hyperinsulinemia[3].

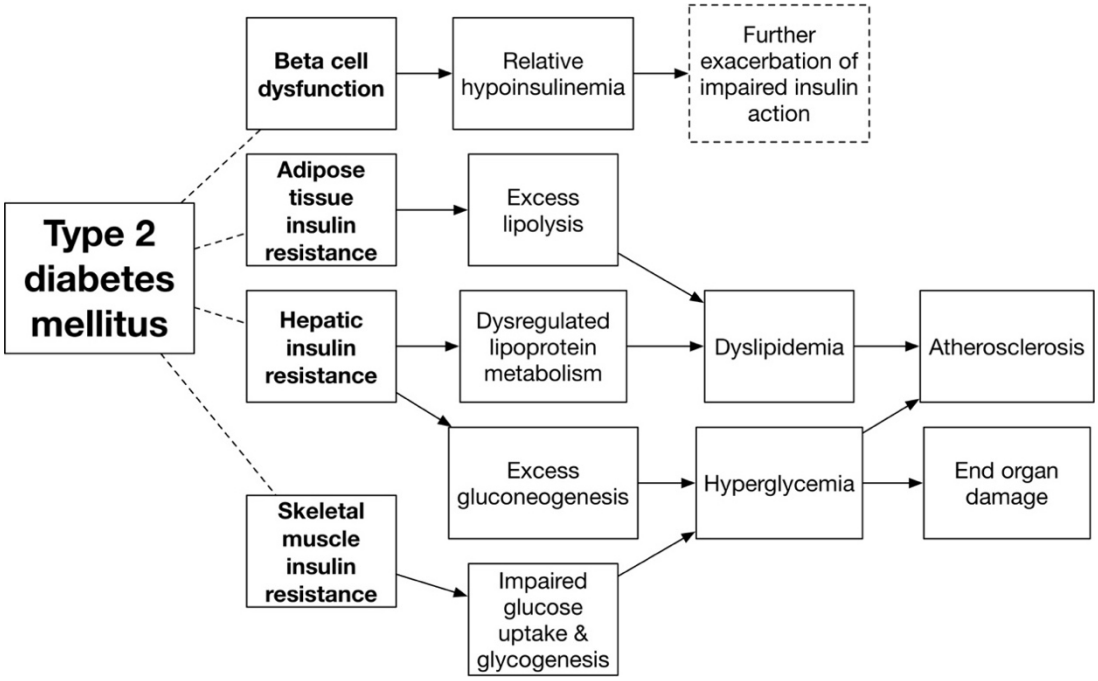

Figure 3 A simplified diagram of the possible mechanisms behind type 2 diabetes mellitus leading to hyperglycemia and dyslipidemia.

Even in the fasted state, individuals with T2DM have alterations in metabolism due to insulin resistance. Adipose tissue lipolysis and release of free fatty acids are increased. Hepatic gluconeogenesis is abnormally elevated in combination with impaired glucose uptake to skeletal muscle, resulting in elevated fasting glucose[3]. After developing insulin resistance, the final step in the pathogenesis of T2DM leading to elevated glucose levels is thought to be the impairment of insulin secretion from ß-cells, as insulin secretion adjusted for the level of insulin resistance (the disposition index) is a strong predictor of developing T2DM[66]. The abovementioned model is a simplified working hypothesis for the pathophysiology of insulin resistance in T2DM. The strict distinction between type 1 diabetes mellitus and T2DM has been questioned as being an oversimplification since different forms of diabetes are combinations of insulin resistance and insulin deficiency, with heterogeneous genetic and pathophysiological backgrounds, and different forms of diabetes often present diagnostic challenges[67]. However, for our purposes T2DM can roughly be considered as a separate entity characterized by the development of insulin resistance due to lifestyle factors and the impairment of insulin secretion that in combination with insulin resistance leads to chronic hyperglycemia. Next, I will briefly review the biology of adipose tissue and, after that, the possible role of SAT dysfunction in the pathogenesis of obesity-related metabolic disease.

2.2.3 SUBCUTANEOUS ADIPOSE TISSUE DYSFUNCTION IN OBESITY

Adipose tissue accounts for around 10-60% of body weight, depending on one's sex and level of obesity. Adipose tissue can be classified into white adipose tissue and brown adipose tissue. Brown adipose tissue is specialized in generating heat through the presence of the protein UCP1 in the mitochondrial membrane, which utilizes the proton gradient across the inner mitochondrial membrane to generate heat[68]. White adipose tissue, by contrast, is dedicated to the storage of energy[69] in the form of triglycerides in the large single lipid droplet that occupies the vast majority of the white adipocyte intracellular space. Recently, the possibility of browning of white adipose tissue, producing "beige" or "brite" adipocytes that are functionally situated between classical brown adipocytes and white adipocytes, has been demonstrated in model organisms[70]. However, our focus will be on SAT, which in humans is considered to consist mainly of white adipocytes and stroma vascular fraction cells such as vascular endothelial cells, fibroblastic preadipocytes, and immune cells[4].

Classically, SAT has been thought to be a passive storage of energy in the form of triglycerides in the lipid droplet that occupies the majority of the adipocyte cytosol[69]. Findings from recent decades have, however, shown that besides being a passive reservoir of triglycerides SAT is an active endocrine organ secreting multiple signal molecules (termed adipokines)[16,71], of which leptin and adiponectin are perhaps the best known. SAT has also been shown to regulate and interact with the immune system, with possible systemic effects through altered secretion of inflammation-related adipokines or cytokines[17,18]. SAT dysfunction has been suggested to lead to lipid overflow[5,72] into ectopic sites, such as the liver, pancreas, heart, muscle, and visceral adipose tissue (VAT), impairing their function and insulin sensitivity through lipotoxicity and other possible mechanisms[3]. Next, I review some of the evidence from model organism and human studies linking the obesity-related SAT dysfunction to systemic metabolic dysregulation and insulin resistance (Figure 4), possibly through increased proinflammatory responses[2,17,18], altered adipokine secretion[4], and lipotoxicity resulting from lipid overflow[3,5,72].

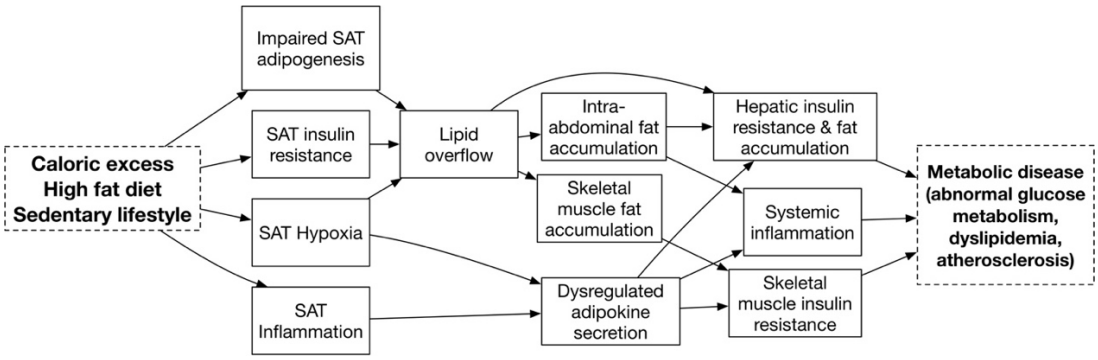

Figure 4 A simplified diagram of the possible mechanisms through which lifestyle factors can cause metabolic disease through their effects on subcutaneous adipose tissue function.

Obesity has been demonstrated to lead to inflammation[2,17,18,73], hypoxia[4], and possibly subsequent insulin resistance[74,75] of SAT. This adipose tissue dysfunction has been thought to produce systemic effects in at least two ways. Inadequate adipogenesis in combination with nutrient excess and impaired ability of insulin to suppress lipolysis in SAT possibly lead to a lipid overflow into other tissues[5,72], which disturbs their function[3]. On the other hand, as the adipose tissue secretes hundreds of different hormone or signal molecules[71], such as leptin, adiponectin, and various cytokines, altered secretion of these adipokines might have systemic effects. Indeed, obesity has been shown to alter the secretion of around 200 different adipokines[71], and adipose tissue inflammation is associated with the secretion of many adipokines (including classical cytokines such as TNF-α, interleukins, and MCP-1)[4]. The altered secretion of these adipokines might have effects on hepatocytes, skeletal muscle cells, and other tissues that drive the metabolic dysfunction associated with obesity and T2DM.

Obesity has been demonstrated to increase SAT inflammation and the recruitment of immune cells (such as macrophages) into the adipose tissue in both rodents and humans[2,17,73]. The presence of inflammatory macrophages in SAT has been demonstrated to cause adipocyte insulin resistance and systemic insulin resistance (probably driven by effects on skeletal muscle)[73]. Mechanistic studies in rodents manipulating the activity of inflammatory pathways in adipose tissue show that increased macrophage-mediated inflammation leads to insulin resistance and decreased macrophage-mediated inflammation leads to insulin sensitivity[73]. Additionally, the correlative studies in humans have shown that weight loss[76] and exercise training[77], both interventions that improve insulin sensitivity, decrease SAT inflammation, whereas infusing free fatty acids, an intervention that increases insulin resistance, induces inflammation[78]. While the word inflammation has a negative connotation, it is not clear whether the inflammatory response in adipose tissue induced by obesity necessarily represents a dysfunctional and harmful response. First of all, adipose tissue macrophages are present roughly

as two phenotypes: proinflammatory M1 macrophages and anti-inflammatory M2 macrophages[17,18], so the presence of macrophages in itself does not imply a proinflammatory process. Second, it has been demonstrated that mouse strains with reduced adipose tissue-specific inflammatory potential also have impaired adipose tissue remodeling and adipogenesis, which lead to increased insulin resistance, ectopic lipid accumulation, and systemic inflammation[79]. This suggests that SAT inflammation could be required for healthy adipose tissue expansion, protecting other tissues from nutrient excess by increasing the storage capacity of SAT, thus making SAT inflammation a putative adaptive response to metabolic stress.

Adipose tissue hypoxia has been suggested to drive some of the metabolic dysfunction associated with obesity[4]. Multiple lines of evidence, although mainly from animal studies, indicate that obesity is associated with an increase in relative adipose tissue hypoxia, which drives changes in adipocyte function and gene expression mainly through the activation of transcription factor HIF-1 (hypoxia-inducible factor 1)[4]. This increasing hypoxia could be driven by the hypertrophy of adipocytes combined with an inadequate increase in adipose tissue vasculature, as it has been demonstrated that despite substantial enlargement of the SAT mass in obesity, the proportion of cardiac output and blood flow to SAT does not increase in humans[80–82]. Adipose tissue hypoxia is associated with changes in the expression of multiple adipokines and increased inflammation[4].

I will next review some of the evidence linking ectopic fat deposition, or lipid overflow from SAT, to metabolic dysfunction. Ectopic fat deposition (the deposition of lipids such as triglycerides in tissues other than the subcutaneous adipose tissue) has been suggested to be a central factor contributing to insulin resistance in obesity and T2DM[3,5,67]. Dysfunctional SAT in the context of caloric excess and a sedentary lifestyle is thought to lead to ectopic fat deposition due to lipid overflow into other tissues such as VAT, liver tissue, epicardial or myocardial tissue, skeletal muscle tissue, and pancreatic tissue[5]. The deposition of lipids in these ectopic sites is thought to interfere with the normal functioning of these tissues, leading to insulin resistance in the liver and skeletal muscle and impaired insulin secretion in the pancreas[3].

Obesity is associated with an increase in adipocyte size in humans[5,24], and adipocyte size seems to be more strongly associated with adipose tissue function than total adiposity or BMI alone[83]. Larger size of SAT adipocytes in humans is associated with insulin resistance[84–87] and is a risk factor for developing T2DM in prospective studies, independent of total adiposity levels[87,88]. The fact that the associations of adipocyte size with insulin resistance and T2DM persist after controlling for total body adiposity[5,83] suggests that adipose tissue function reflected in adipocyte size is involved in the pathogenesis of metabolic dysfunction associated with obesity. Hypertrophied adipocytes have higher rates of lipolysis in basal and stimulated conditions[89], and studies show that hypertrophied adipocytes are

more insulin-resistant[90] and display lower uptake of free fatty acids from the circulation[83], thus adipocyte size seems to be involved in the pathogenesis of metabolic dysfunction in obesity.

Additionally, obesity-related SAT mitochondrial dysfunction has been implicated as a possible factor leading to impaired adipogenesis and increased SAT inflammation[23]. Obesity has been demonstrated to be associated with lower SAT PGC-1α expression[22,91,92] (an important regulator of mitochondrial biogenesis), lower mitochondrial DNA content[22], lower mitochondrial oxidative phosphorylation-related gene expression and protein amount[22], lower expression of other mitochondrial metabolic pathways[25,93], and lower mitochondrial oxidative activity[94]. Interestingly, SAT mitochondrial BCAA (branched chain amino acid) catabolism pathway expression has also been shown to be correlated with liver fat amount[25], possibly linking ectopic fat deposition to SAT mitochondrial function, although both of them might just as well be markers for the severity of obesity.

The obesity-associated changes in adipose tissue function due to mitochondrial dysfunction, inflammation[2], hypoxia[4], and hypertrophy of the adipocytes[83] that seem to impair lipid uptake and storage in SAT might contribute to lipid overflow into other tissues, impairing their function[3,5,72]. This explanation for the relevance of adipose tissue function in determining whole-body metabolic derangements has been called the "lipid overflow hypothesis"[5]. Multiple lines of evidence implicate impaired SAT fat storage and ectopic fat deposition as a possible causal mechanism in obesity-induced insulin resistance and metabolic dysfunction. Firstly, animal models of lipodystrophy and clinical observations of human lipodystrophy patients suggest that impaired fat storage and subsequent ectopic fat accumulation lead to insulin resistance and dyslipidemia[95]. Secondly, intramyocellular lipids in skeletal muscle are associated with insulin resistance in humans[96,97], and a mechanism linking intramyocellular diacylglycerols to muscle insulin resistance has been demonstrated[3]. Thirdly, hepatic insulin resistance is closely related to hepatic lipid content in humans[98], reversal of hepatic steatosis due to moderate weight loss improves hepatic insulin sensitivity in humans[99], and a mechanism linking hepatic diacylglyceroles to hepatic insulin resistance has been demonstrated[100]. However, in humans multiple genetic mutations resulting in hepatic steatosis do not cause hepatic insulin resistance[101], which casts some doubt on the assertion that hepatic steatosis is the causal mechanism behind hepatic insulin resistance, without considering the type of steatosis. Relatedly, results from a study comparing individuals with ordinary hepatic steatosis and individuals with hepatic steatosis due to the I148M variant of the *PNPLA3* gene, suggest that ordinary metabolic hepatic steatosis is connected to insulin resistance through increased amounts of saturated fat and ceramides in the liver lipidome, since *PNPLA3* hepatic steatosis, where the steatosis is characterised by increased polyunsaturated triacylglycerols, does not seem to increase insulin resistance[102]. Additionally, exposure of pancreatic ß-cells to free fatty acids has been demonstrated to lead

to impaired insulin secretion[103,104], possibly linking lipid overflow with the impaired insulin secretion observed in T2DM[105].

While the ectopic accumulation of lipids into hepatic and skeletal muscle can be somewhat straightforwardly linked to metabolic health, VAT accumulation is also strongly associated with metabolic health in obesity[5]. Although subcutaneous adipose tissue accumulation itself is seen as relatively benign, the accumulation of VAT has been strongly linked to metabolic dysfunction[5]. The term VAT is technically used to refer to adipose tissue within the thoracic (e.g. epicardial fat) and abdominal cavity[106], but we use it here to refer only to adipose tissue within the abdominal cavity: the intraperitoneal adipose tissue in the greater omentum and the mesentery and extraperitoneal adipose tissue in the retroperitoneum, as is common practice[5]. Although the intraperitoneal adipose tissue probably has a different relevance to metabolic health than retroperitoneal fat, since adipose tissue in the greater omentum and mesenterium are drained through the portal vein to the liver, in this thesis they are not considered separately[5]. Visceral obesity is strongly associated with insulin resistance, T2DM, and dyslipidemia, however, as with other ectopic fat deposits, it is unclear whether the VAT has a causal role or not[5]. Tchernof & Després[5] outline three scenarios that explain why VAT accumulation is associated with metabolic complications. In the first scenario, as VAT is hyperlipolytic and resistant to the suppression of lipolysis by insulin, this leads to the liver being exposed to an excess of nonesterified fatty acids, which might lead to impairments in liver metabolism, resulting in increasing dyslipidemia, increased hepatic glucose production, and reduced hepatic insulin clearance. In the second scenario, as VAT is more prone to inflammation and to the accumulation of macrophages, VAT dysfunction could lead to increased systemic inflammation, resulting in increased insulin resistance in other tissues. In the third scenario, visceral adiposity is a consequence of SAT dysfunction and inability of SAT to store lipids in the context of nutrient excess, which leads to lipid overflow that is buffered by the VAT, and thus, VAT could protect other ectopic sites (e.g. skeletal muscle and the liver) from lipotoxicity[5]. A fourth possible explanation would be that VAT accumulation is merely associated with metabolic dysfunction, without having a causal role in exacerbating or protecting from metabolic dysfunction[107].

In summary, lifestyle factors might lead to SAT dysfunction and subsequent systemic metabolic dysfunction by at least two mechanisms: 1) SAT inflammation and hypoxia lead to dysregulated adipokine secretion and 2) SAT mitochondrial dysfunction, insulin resistance, hypoxia, and impaired adipogenesis lead to lipid overflow. Both mechanisms could potentially lead to ectopic fat accumulation, systemic inflammation, and insulin resistance of other tissues, exacerbating the metabolic dysfunction associated with obesity (Figure 4).

2.2.4 WEIGHT LOSS, EXERCISE, AND METABOLIC HEALTH

Next, I will briefly review how dietary and exercise-related lifestyle interventions might affect metabolic health. Weight loss through reduced caloric intake or increased energy expenditure through exercise is generally recommended to treat obesity and metabolic syndrome[108]. Increasing physical activity, even without an overt goal of weight loss, is generally recommended and has been shown to improve glycemic control, reduce VAT, and reduce plasma triglycerides in people with T2DM even without weight loss[109]. Lifestyle modification programs aimed at producing moderate weight loss by dietary changes and increasing physical activity have also been demonstrated to improve glucose tolerance and prevent T2DM in subjects with impaired glucose tolerance in multiple high-quality randomized controlled trials[110–114]. There are many possible mechanisms by which weight loss and increased physical activity lead to improved metabolic health in obesity and T2DM. Reduced SAT dysfunction has been proposed to mediate at least some of the benefits of weight loss[115] and exercise[116,117].

Interventions in humans leading to marked weight loss (e.g. due to bariatric surgery) have been shown to reduce systemic inflammatory markers, SAT macrophage inflammation, and SAT inflammatory gene expression[76,118–120]. Additionally, in rodents, the manipulation of adipocyte-specific metabolic pathways known to be affected by diet-induced weight loss in humans[115] have been shown to influence whole-body insulin sensitivity, glucose tolerance, and lipid metabolism[121–125], suggesting that improvement of SAT function has a causal role in determining beneficial effects of weight loss.

However, whether this change in systemic and SAT inflammatory markers is causally related to the changes in insulin sensitivity during weight loss is unclear. Magkos et al.[115] studied the effects of a dietary weight loss intervention aiming at different levels of weight loss (weight maintenance, 5.1% weight loss, 10.8% weight loss, and 16.4% weight loss) on adipose tissue function and metabolic health in sedentary obese subjects with BMIs of around 38 kg/m^2. Weight loss of 5% did not have significant effects on 2-hour plasma glucose or total glucose AUC in an OGTT (oral glucose tolerance test). However, a hyperinsulinemic-euglycemic clamp with infusion of isotopically labeled tracers showed increased adipose tissue insulin sensitivity (insulin-mediated suppression of palmitate rate of appearance in plasma), liver insulin sensitivity (insulin-mediated suppression of glucose rate of appearance in plasma), and skeletal muscle insulin sensitivity (insulin-mediated stimulation of glucose rate of disappearance from plasma). With additional weight loss of 11-16%, only the muscle insulin sensitivity of the tissue-specific measures was increased further. Surprisingly, 5% weight loss did not affect systemic or local SAT markers of inflammation, but progressive weight loss to 11-16% led to a downregulation of SAT inflammation-related genes. The results of this dietary weight loss study suggest that while a moderate weight loss of 5% improves tissue-specific insulin sensitivity in SAT, liver, and skeletal muscle, this initial improvement is not accompanied by a decrease in systemic or inflammatory

markers, and thus, is perhaps independent of systemic and adipose tissue inflammation. Conversely, a weight gain of 5-6% is associated with decreasing insulin sensitivity, without an increase in SAT or systemic markers of inflammation in two studies on humans[126,127]. However, the statistical power in these three studies[115,126,127] showing the dissociation of changes in insulin sensitivity and changes in systemic or SAT inflammation is limited, with only 6-19 subjects in each group, and thus, the they cannot reliably rule out meaningful medium-sized or smaller effects.

In summary, weight loss and increased physical activity counteract the metabolic dysfunction associated with obesity and T2DM. While evidence from animal studies shows that SAT and systemic inflammation could be causally linked to insulin resistance, small studies on weight loss[115] and weight gain[126,127] in humans suggest that changes in insulin resistance occur even without an observed change in SAT or systemic inflammation, suggesting that rather than inflammation other mechanisms, such as alterations in adipokine secretion and lipid overflow, could underlie the changes in insulin resistance due to weight loss or weight gain in humans.

2.3 NAD^+/SIRT PATHWAY AND METABOLIC HEALTH

2.3.1 SIRTUIN PROTEINS

A potential mechanism involved in SAT dysfunction in overnutrition or obesity is downregulation of the NAD^+/SIRT pathway, which is an intracellular energy-sensing mechanism that regulates various cellular functions according to NAD^+ levels[19–21,128]. The mammalian sirtuins (or SIRTs) are a protein family comprising seven different proteins (SIRT1-7) expressed in various metabolically active tissues, including adipose tissue, muscle tissue, and hepatic tissue[20]. Different sirtuins have various enzymatic activities, and NAD^+-dependent deacetylation is perhaps the best characterized function of sirtuins[129]. Sirtuins regulate cellular functions by deacylating various different target proteins such as histones, transcription factors, and enzymes[129]. The deacylation of lysine residues of target proteins in the sirtuin reaction uses NAD^+ as a cosubstrate and converts it to nicotinamide and O-acetyl-ADP-ribose, and thus, the deacylation activity of sirtuins is regulated or determined by cellular NAD^+ levels[129]. As cellular NAD^+ levels reflect the cellular energy levels, the NAD^+ dependence of sirtuins makes them act as metabolic sensors that regulate cellular function accordingly[19]. For example, in model organisms NAD^+ levels in muscle, liver, and adipose tissue rise during caloric restriction[130] and at least in skeletal muscle after exercise[131].

Research interest in sirtuin proteins started from the observation that in budding yeast the overexpression of the sirtuin homolog *SIR2* resulted in an extension of the yeast lifespan[129,132]. Since then, there have been hundreds of

studies in various model organisms on the role of sirtuins in longevity and metabolic health[129]. Additionally, multiple SIRT1-activating compounds (STACs) have been discovered and studied extensively[133]. There are currently multiple phase I, II, and III clinical trials on STACs (resveratrol, SRT2104, and nicotinamide riboside) for the treatment of cardiovascular disease and diabetes in humans[129].

SIRT1 and SIRT3 are the most studied and prominent sirtuins and perhaps the most important sirtuin proteins with regard to metabolism in mammals[21]. SIRT1, localized in the nucleus and the cytosol[19], regulates several regulatory proteins involved in development, energy metabolism, inflammation, and DNA repair[21]. Out of the known targets for SIRT1, especially relevant for the metabolic function of adipose tissue are PPAR-γ, NF-κB, PGC-1α, and HIF-1. PPAR-γ is a nuclear receptor that is considered to be one of the master regulators of adipocyte differentiation as it promotes adipogenesis[21], and during fasting SIRT1 has been demonstrated to inhibit adipogenesis and stimulate lipolysis and mobilization of free fatty acids from white adipose tissue by repressing the activity of PPAR-γ[134]. NF-κB is a transcription factor that regulates various cellular functions, including immune response, inflammation, and apoptosis[135]. SIRT1 has been shown to interact with and deacetylate the NF-κB complex[136] and to reduce NF-κB activity[137]. PGC-1α is an important regulator of mitochondrial biogenesis, and SIRT1 has been demonstrated to interact with and deacetylate PGC-1α, leading to the increased transcription of the downstream pathways controlling mitochondrial gene expression[138]. HIF-1 is a transcription factor that mediates cellular responses to hypoxia by regulating the transcription of hundreds of genes[139]. SIRT1 has been demonstrated to inactivate HIF-1, thus modulating the cellular responses to hypoxia[140]. While the list above is not exhaustive regarding relevant SIRT1 targets, it provides possible mechanistic links between SIRT1 activity and adipose tissue dysfunction relevant to obesity and metabolic disease, since inadequate adipogenesis[5], inflammation[18], impaired mitochondrial biogenesis[22], and hypoxia[4] of the adipose tissue have all been proposed as possible mechanisms in the pathogenesis of obesity-related metabolic disease (Figure 4).

SIRT3 is localized in the mitochondria[19] and regulates the expression of several mitochondrial proteins and the activity of several mitochondrial enzymes[21], promoting fatty acid oxidation[141] and increasing antioxidative capabilities in mitochondria[142]. Additionally, SIRT3 increases PGC-1α expression, possibly promoting mitochondrial biogenesis[141]. NAD^+ boosters[128], SIRT1[128], and SIRT3[143] all induce the mitochondrial unfolded protein response (UPR^{mt}), which is a mitochondrial protein homeostasis pathway that protects mitochondria from the proteotoxic stress caused by misfolded proteins[144]. UPR^{mt} activation has been shown to promote longevity in *Caenorhabditis elegans*[128,145]. Less is known about the function of other sirtuin proteins relevant to metabolism. SIRT5 is a mitochondrial sirtuin that regulates the lysine acylation of mitochondrial proteins[146], and possibly

increases mitochondrial fatty acid oxidation[147] and respiration[148]. SIRT2, a cytosolic and nuclear sirtuin[20], seems to promote adipogenesis and lipolysis in adipose tissue through activating FOXO1[149,150].

In summary, sirtuin proteins are NAD^+-dependent enzymes that control the actions of various cellular proteins (e.g. transcription factors, enzymes, and histones) through their deacylase activity. This activity of sirtuins is linked to cellular energy levels or metabolic state reflected in the NAD^+ levels. Overall, sirtuins, of which SIRT1 is most studied, seem to affect adipose tissue function in favorable ways regarding metabolic health.

2.3.2 REGULATION OF SIRTUIN EXPRESSION AND ACTIVITY

Next, I will briefly review the factors affecting sirtuin (or SIRT1) expression and activity. According to promoter analysis of *SIRT1*, multiple transcription factors related to metabolism enhance *SIRT1* expression (FOXO1, PPARα, PPARß, CREB) or repress it (PPAR-γ, CHREBP, PARP2, HIC1)[19], although to my knowledge only CREB, ChREBP, and PARP2 have been demonstrated to control *SIRT1* expression *in vivo*[151,152]. CREB (cAMP response element-binding protein) is a transcription factor that mediates a response to low nutrient availability[153], whereas ChREBP (carbohydrate-responsive element-binding protein) is a transcription factor that mediates effects of high nutrient availability on cellular metabolism, shifting it towards energy storage and usage[154]. Noriega et al.[151] have shown that under low nutrient availability CREB downregulates *SIRT1* expression, whereas with high nutrient availability ChREBP upregulates *SIRT1* expression, providing a possible mechanistic link between obesity and lowered SAT *SIRT1* expression. An additional link between obesity and SAT *SIRT1* expression comes from the observation that *Sirt1* expression is increased by increasing NAD^+ levels[155] coupled with the observation that NAD^+ levels and the NAD^+/NADH ratio increase in mouse adipose tissue during caloric restriction[130]. Nevertheless, to my knowledge there is no direct evidence for what causes low SAT *SIRT1* expression in obesity. In theory, high nutrient intake or caloric excess related to obesity might lower SAT *SIRT1* expression through lowering adipocyte NAD^+ levels[155] and activating adipocyte ChREBP[151] (Figure 5).

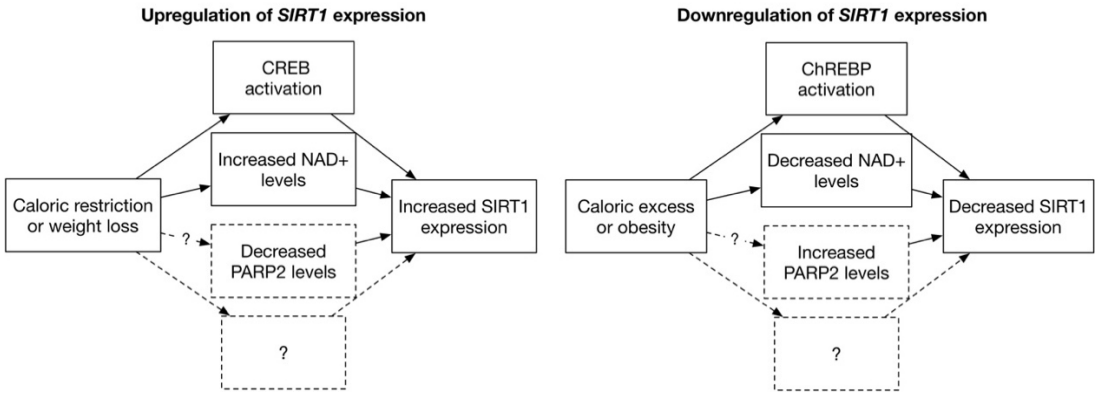

Figure 5 Possible mechanisms for the regulation of SAT *SIRT1* expression in response to lifestyle factors.

SAT *Sirt1* expression has been demonstrated to be increased by caloric restriction in both rats[156] and mice[130], whereas high-fat feeding decreases SAT *Sirt1* expression in mice[157]. Also, in mouse SAT, high-fat feeding causes the inflammation-induced cleavage of SIRT1 proteins by Caspase-1, making the cleaved SIRT1 less active and less stable[157]. In humans, SAT *SIRT1*[158–162], *SIRT3*[160], and *SIRT6*[160] expression have been demonstrated to be negatively associated with BMI. SAT *SIRT1* expression has also been demonstrated to be lower in obese (BMI ~ 37 kg/m²) individuals with higher amounts of VAT than individuals of equal BMI with lower VAT amount[163]. Additionally, in a study of 9 obese (BMI ~ 46 kg/m²) nondiabetic women, total fasting for 6 days significantly increased SAT *SIRT1* expression. In a study of obese (BMI ~ 43 kg/m²) subjects undergoing laparoscopic adjustable gastric banding, SAT *SIRT1*, *SIRT3*, and *SIRT6* expressions were significantly increased at 6 months after the procedure concomitant to a mean 21.5 kg decrease in body weight[160]. Also, circulating plasma SIRT1 amount increases after intragastric balloon-induced weight loss in humans[164].

SIRT1 activity is controlled via post-translational modifications (e.g. phosphorylation), complex formation with other proteins, and at the substrate level by NAD^+ availability[19]. While post-translational modifications and interactions with other proteins possibly play important roles in regulating SIRT activity, they are not further examined in this thesis. The availability of NAD^+ in cells is determined by its biosynthesis and consumption. NAD^+ can be synthesized from nicotinic acid, nicotinamide riboside, or *de novo* from tryptophan[26] (Figure 6). Importantly, the nicotinamide (NAM) produced by enzymes using NAD^+ as a substrate (mainly SIRTs and PARPs) can be salvaged back into NAD^+ through the salvage pathways[26] (Figure 6C). In mammals, the salvage pathways seem to be the most important source of intracellular NAD^+[26]. NAD^+ salvage from NAM comprises two steps: first NAMPT (nicotinamide phosphoribosyltransferase) metabolizes NAM into NAM mononucleotide, then NMNAT1, 2, or 3 (nicotinamide mononucleotide adenylyltransferase 1-3) converts NAM mononucleotide into NAD^+[26].

NAMPT is the rate-limiting step in this pathway[165], and thus, probably the most important biosynthetic enzyme determining NAD^+ levels in mammals. Interestingly, SAT *NAMPT* expression has been observed to be negatively associated with BMI in humans[166,167], which suggests that obesity decreases SAT adipocyte intracellular NAD^+, and subsequently, *SIRT1* expression at least in part through downregulating *NAMPT* expression, which reduces regeneration of NAD^+ through the salvage pathways. The NMNAT enzymes could also be considered as important enzymes in the biosynthesis of NAD^+ since they are involved both in the Preiss-Handler pathway (Figure 6B) and the salvage pathways (Figure 6C).

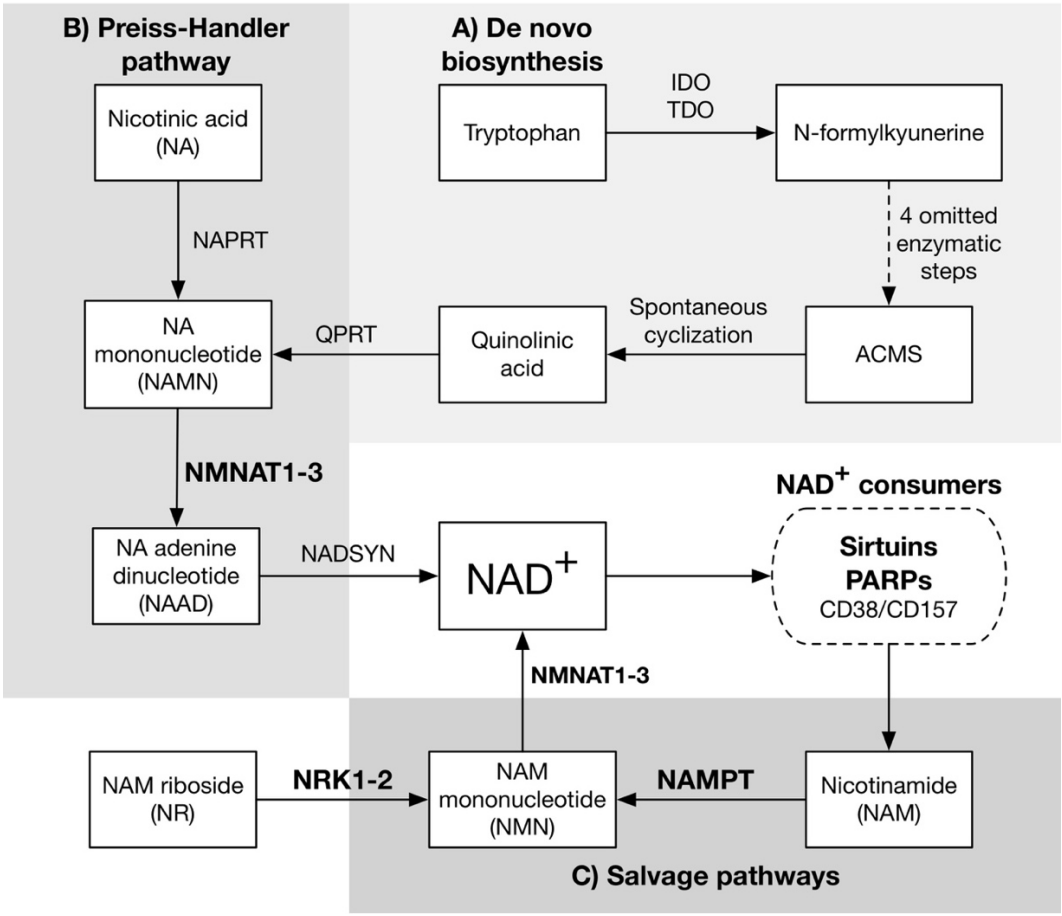

Figure 6 Outline of the NAD^+ synthesis pathways: A) *de novo* biosynthesis from tryptophan, B) the Preiss-Handler pathway, and C) the salvage pathways. Enzymes or proteins that are the focus of this thesis are shown in bold font. Cofactors (such as ATP) have been left out of the diagram. ACMS, a-amino-b-carboxymuconate-ε-semialdehyde; IDO, indoleamine 2,3-dioxygenase; NA, nicotinic acid; NADSYN, NAD^+ synthetase; NAMN, NA mononucleotide; NAMPT, nicotinamide phosphoribosyltransferase; NAPRT, NA phosphoribosyltransferase; NMN, NAM mononucleotide; NMNAT, NMN adenylyltransferase; NR, nicotinamide riboside; NRK, NR kinase; PARP, poly ADP ribose polymerase, QPRT, quinolinate phosphoribosyltransferase; TDO, tryptophan 2,3-dioxygenase. Adapted from Cantó, Menzies, & Auwerx[26].

PARPs (poly ADP ribose polymerases) are important consumers of intracellular NAD^+ and compete with sirtuins for the same NAD^+ pool[26], thus indirectly repressing sirtuin activity by making less NAD^+ available to sirtuins[168]. Several physiological stimuli, such as DNA damage, oxidative stress, and aging, stimulate PARPs[26], but even high-fat feeding has been shown to induce PARP activity in mouse muscle[168]. Recently, PARPs have been implicated in playing a role in regulating metabolism since deletion of *Parp1*[168] and *Parp2*[169] or PARP inhibition[170] have been demonstrated to protect mice from high-fat feeding-induced obesity and insulin resistance, and to activate SIRT1.

In summary, obesity is associated with low SAT SIRT1, SIRT3, and SIRT6 expression in humans, and weight loss has been shown to lead to increased expression of these SIRTs in SAT. The obesity-related decrease in SAT SIRT1 expression could be due to reduced NAD^+ availability and/or increased ChREBP activity. SIRT activity is regulated by 1) intracellular NAD^+ availability, which is mainly determined by NAD^+ synthesis or regeneration through the salvage pathways (limited by NAMPT), 2) competing usage of NAD^+ (mainly by PARPs), and 3) post-translational modifications and protein interactions.

2.3.3 SIRTUINS AND METABOLIC HEALTH IN MODEL ORGANISMS

Although the effect of sirtuin overexpression or activation on lifespan in mammals has been questioned[19], results from some animal studies suggest that increased SIRT1 protein synthesis and activity might mediate the effects of caloric restriction on metabolism and increased lifespan[156,171–173]. First of all, *Sirt1*$^{-/-}$ knockout mice do not increase their lifespan on caloric restriction as *Sirt1*$^{+/-}$ or *Sirt1*$^{+/+}$ mice do[171]. In some studies, when mice are treated with the STACs (SIRT1-activating compounds) resveratrol[173] or SRT1720[172] they only seem to increase their lifespan on high-fat feeding regimens. However, some more recent studies have demonstrated an increased lifespan in mice on normal chow treated with STACs[174,175].

Even if sirtuin activation does not ultimately increase lifespan in mammals living in a healthy environment, they might have a significant role in protecting metabolic health in the context of caloric excess or other metabolic stressors[19]. Along these lines, multiple studies on rodents show that the overexpression of *Sirt1* or administering different STACs improves metabolic health parameters and protects from metabolic derangements due to high-fat feeding[170,172,176–178]. Lagouge et al.[178] have shown that supplementing resveratrol, a known STAC[133], protects mice from weight gain due to high-fat feeding, increases their insulin sensitivity and glucose tolerance, leads to smaller adipocyte size in SAT, and increases their BAT mitochondrial activity. Bordone et al.[176] have shown that mice overexpressing *Sirt1* are leaner, have better glucose tolerance, and perform better on a test of motor function (rotarod test). Feige et al.[177]

demonstrated that giving mice SRT1720, a specific STAC[133], prevents weight gain due to high-fat feeding, without differences in feeding behavior or increased locomotor activity, by increasing total body oxidative metabolism. The SRT1720 treatment also improved glucose tolerance and insulin sensitivity with both high-fat feeding and normal chow feeding. The treated mice developed less white adipose tissue and the adipocyte size within white adipose tissue was smaller[177]. Despite SIRT1 being a known repressor of PPAR-γ[134], an important transcription factor inducing adipogenesis, there were no changes in the expression of PPAR-γ downstream targets[177], suggesting that SIRT1 activation did not impair adipogenesis and the ability of adipocytes to store lipids. SRT1720 did, however, increase the expression of hormone-sensitive lipase (LIPE)[177], indicative of increased lipolysis. Pirinen et al.[170] have shown that administering PARP inhibitors, which increase cellular NAD^+ and subsequently activate SIRTs, to mice prevents weight gain due to high-fat feeding, increases mitochondrial oxidative capacity, and increase their exercise capacity. Additionally, they showed that PARP inhibitors improve mitochondrial function in primary myotubes of obese humans, possibly through SIRT1 activation[170]. Results by Li et al.[179] suggest that resveratrol administration decreases adipose tissue HIF-1 activity in mice on a high-fat diet, and they showed that resveratrol reduces HIF-1 accumulation in cultured adipocytes. Liu et al.[180] showed that resveratrol administration to mice on a high-fat diet led to decreased adipose tissue inflammation through increased SIRT1 expression or activation. These studies on *Sirt1* overexpression and STAC administration are not, however, adipose tissue-specific, and the effects could be mediated through overexpression or SIRT activation in muscle and liver tissue, among others. Nevertheless, there is at least one study by Xu et al.[181], where mice overexpressing human *SIRT1* in an adipose tissue-specific manner were evaluated. Raised on normal chow, the SAT *SIRT1* overexpressing mice had lower fat %, less VAT, lower ectopic liver and muscle triglyceride deposition, and better systemic insulin sensitivity than wild-type mice[181]. Moreover, SIRT3 has been suggested to protect metabolic health in obesity by improving mitochondrial function and increasing energy expenditure[141,182].

In addition to studies of sirtuin overexpression or activation, some researchers have investigated the effects of knocking down *Sirt1* or decreasing its activity. Chalkiadaki and Guarente[157] studied mice with adipose tissue-specific deletion of *Sirt1*, and showed that on a normal chow diet they gain more weight and develop larger and more hypertrophied adipocytes in SAT than their wild-type counterparts. On a normal chow diet, the 16-week-old SAT *Sirt1* knockout mice had similar glucose tolerance and insulin sensitivity to the wild-type mice. However, after being on a high-fat diet for 12 weeks the knockout mice developed more insulin resistance and were more glucose-intolerant than the wild-type mice. Additionally, at an age of one year, the adipose tissue *Sirt1* knockout mice fed normal chow were more insulin-resistant and glucose-intolerant than wild-type mice[157]. These results suggest

that lack of adipose tissue SIRT1 makes mice more metabolically susceptible to a high-fat diet, but even on normal chow they develop insulin resistance as they age. Interestingly, the *Sirt1* knockout mice on a normal chow diet had similar gene expression patterns in SAT as the wild-type mice on a high-fat diet[157], suggesting that loss of SIRT1 activity has similar effects on adipose tissue function as high-fat feeding. Xu et al.[181] studied transgenic mice with decreased adipose tissue-specific SIRT1 activity fed *ad libitum* with normal chow and showed that the mice with decreased adipose tissue SIRT1 activity had lower systemic insulin sensitivity and glucose tolerance and higher ectopic fat deposition of triglycerides into muscle and liver tissue. Mayoral et al.[183] also studied adipose tissue-specific *Sirt1* knockout mice fed *ad libitum*. On normal chow, the knockout mice were more insulin-resistant and glucose-intolerant than wild-type control mice. Also after 5 weeks of high-fat feeding, the knockout mice were more insulin-resistant and glucose-intolerant than the wild-type controls. However, after 15 weeks of high-fat feeding the situation reversed: the *SIRT1* adipose tissue knockout mice were relatively more insulin-sensitive and glucose-tolerant than wild-type controls. Thus, the knockout mice developed insulin resistance and glucose intolerance earlier than wild-type controls, but these changes plateaued in the knockout group, and after 15 weeks of high-fat feeding the metabolic dysfunction in wild-type mice surpassed that of the knockout mice. Mayoral et al. [183] explain the better relative insulin sensitivity and glucose tolerance observed in the adipose tissue *SIRT1* knockout mice after 15 weeks of chronic high-fat feeding with the fact that they exhibited hyperacetylation of adipose tissue PPAR-γ, which leads to its activation and possibly subsequent insulin sensitization and increased adipogenesis. All of the three studies presented in this paragraph indicate that reducing adipose tissue-specific *Sirt1* expression or activity leads to systemic insulin resistance and glucose intolerance during 5-12 weeks of high-fat feeding. However, Mayoral et al.[183] observed that 15 weeks of high-fat feeding led to a more insulin-sensitive and glucose-tolerant phenotype than in wild-type mice, indicating that the relationship between knocking out adipose tissue *Sirt1* and insulin sensitivity during high-fat feeding in rodents is complex.

Because postprandial or OGTT glucose uptake and insulin sensitivity are mainly determined by the actions of insulin on skeletal muscle[184], it is not clear why decreased SIRT1 activity in SAT would affect insulin sensitivity. However, in obese individuals, adipose tissue might play larger role in glucose uptake relative to skeletal muscle. Dadson et al.[185] studied obese patients undergoing bariatric surgery and lean controls using a hyperinsulinemic euglycemic clamp and PET imaging to determine glucose uptake to different tissue sites. They demonstrated that the proportion of total glucose uptake into visceral and subcutaneous fat, relative to glucose uptake into muscle, is higher in obese individuals than in leaner controls. With these results in mind, it could be that adipose tissue insulin sensitivity, possibly regulated by SIRT1 activity, might play a larger role in determining whole body insulin sensitivity or glucose

tolerance in obese individuals than in leaner individuals where skeletal muscle is more central. Moreover, results from Xu et al.[181] showing increased ectopic triglyceride deposition into muscle and liver tissue suggest a possible mechanism, as ectopic muscle fat deposition has been demonstrated to lead to muscle insulin resistance[3]. Also the cross-talk between adipose tissue and skeletal muscle tissue mediated by insulin-sensitizing adipokines, such as adiponectin and FGF21[4], might also explain this connection, as SIRT1 has been demonstrated to regulate their expression[21]. Similar mechanisms might be behind the association between STAC administration or *Sirt1* overexpression and insulin sensitivity, although their direct effects on skeletal muscle cells probably also contribute.

In summary, mouse studies show that global or adipose tissue-specific *Sirt1* overexpression generally leads to leaner, insulin-sensitive, and glucose-tolerant phenotypes and protects from metabolic dysfunction due to high-fat feeding. Similar effects are observed after pharmacologic SIRT1 activation with STACs. Transgenic mice with adipose tissue-specific knockout Sirt1 or decreased SIRT1 activity generally show the opposite effects.

2.3.4 SIRTUINS AND METABOLIC HEALTH IN HUMAN STUDIES

Next, I review some human studies on the role of sirtuin expression and pharmacologic activation. Studies in humans on the relevance of SAT SIRT1 in metabolic health are scarce. SAT *SIRT1* expression has been demonstrated to be correlated with whole-body insulin sensitivity determined with a hyperinsulinemic-euglycemic clamp[186]. The SAT expression of *PGC-1α* and several other SIRT1 target genes has been shown to be associated with whole-body energy expenditure during fasting and during hyperinsulinemic-euglycemic clamp[186]. In addition, three *SIRT1* single-nucleotide polymorphisms have been associated with whole-body energy expenditure during fasting and hyperinsulinemic-euglycemic clamp[178]. Regarding studies on SAT inflammation and SIRTs, SAT *SIRT1* expression has been shown to be correlated with SAT expression of macrophage markers[159]. In a study grouping healthy obese (BMI ~ 37 kg/m^2) individuals into two groups of high and low VAT/(VAT+SAT) ratios, the group with higher VAT amount had significantly lower *SIRT1* expression in SAT, more SAT macrophage infiltration, and higher inflammatory gene expression in SAT[163]. Additionally, the extent of macrophage infiltration in SAT correlated negatively with SAT *SIRT1* expression[163].

While findings from animal studies administering resveratrol and other STACs show benefits regarding metabolic health and insulin sensitivity, clinical trials in humans do not always show benefits. In a meta-analysis of six small randomized controlled clinical trials with a total of 192 type 2 diabetes patients, resveratrol supplementation significantly improved HbA1c, but not HOMA-IR, fasting glucose, triglyceride, LDL, or HDL levels[187], although some

heterogeneity existed in the studies. The variable results on the efficacy of resveratrol supplementation in humans could be explained by differences in disease severity of the patients or variability in the bioavailability of resveratrol supplements[129]. There are two phase II clinical trials on SRT2104, a SIRT1-specific STAC, administering SRT2104 to type 2 diabetic subjects (NCT01018017 and NCT00937326). At the time of writing, both were completed, but only one of them had its results published. Baksi et al.[188] studied around 42 subjects in each study arm: placebo, 0.25 g/day, 0.5 g/day, 1.0 g/day, and 2.0 g/day of SRT2014 oral supplementation for 28 days. There was significant pharmacokinetic variability and plasma SRT2014 levels were not dose-proportional and were lower than intended compared with levels found efficacious in mouse studies. There were no clear dose-response effects on fasting glucose or insulin, postprandial glucose or insulin, or HbA1c. However, there was a significant weight reduction of 1.5 kg at 28 days in the 2.0 g/day arm relative to placebo and some modest improvements in the lipid profile[188]. It remains to be seen whether SRT2014 will prove efficacious in improving glucose tolerance and insulin sensitivity in type 2 diabetic patients if the pharmacokinetic problems observed in Baksi et al.[188] are solved. Two phase I clinical trials on SRT2014 supplementation in healthy elderly subjects with 20-24 participants in each arm have observed significant decreases in body weight, LDL, and triglyceride levels in the SRT2014 arms relative to placebo[189,190]. One of these studies also looked at changes in OGTT responses, but found no significant differences between the SRT2014 and placebo groups in glucose tolerance or insulin sensitivity[189], although the study subjects were healthy without overt type 2 diabetes, and the study has insufficient statistical power to rule out meaningful effects.

In summary, there is some evidence in humans linking SAT SIRT1 expression and SIRT1 single-nucleotide polymorphisms with whole-body energy expenditure during fasting and during a hyperinsulinemic-euglycemic clamp test. While studies on SIRT1 activating compounds in animals are promising, initial small clinical trials in humans on resveratrol or SRT2014 supplementation have not shown clear improvements in insulin sensitivity or glucose tolerance, although some improvements in lipid profiles have been observed.

2.4 CARDIORESPIRATORY FITNESS AND VO_{2MAX}

2.4.1 PHYSIOLOGY OF CARDIORESPIRATORY FITNESS

While in layman's terms cardiorespiratory fitness (CRF) could be thought of as corresponding to one's ability to perform well in a physically strenuous task, such as running a marathon or running on a treadmill (exercise capacity), the exact definition and measurement of CRF for the purposes of physiology are

more complicated. In clinical physiology, CRF is usually defined as the level of maximal oxygen uptake during exercise (VO_{2max})[191], generally expressed in liters or milliliters of O_2 per minute. The gold standard measure of VO_{2max} is a graded exercise test combined with direct measurement of O_2 uptake and CO_2 production from inspired and expired air[192]. Spiroergometry is one such test. While there is variation in different spiroergometry protocols, the following protocol illustrates the method. The subject begins cycling on a cycle ergometer at a light workload. The workload is then raised in intervals until exhaustion or when the respiratory quotient VCO_2/VO_2 (the ratio of exhaled CO_2 to inspired O_2) is raised above 1.1, indicative plateauing of O_2 uptake and CO_2 accumulation. The VO_2 (oxygen uptake) before termination of the test corresponds to the VO_{2max}[47]. While VO_{2max} is a relatively well-defined and unambiguous measure, its physiological basis and interpretation require some discussion.

VO_{2max} [mL_{O2}/min] during maximal exertion follows from the following equation: maximal cardiac output (Q_{max}, [L_{blood}/min]) times the maximal arteriovenous oxygen content difference (Ca-vO_{2max}, [mL_{O2}/L_{blood}])[193]. Q_{max} can be expressed as maximal stroke volume (SV_{max}) times the maximal heart rate (HR_{max}). So the equation determining VO_{2max} is:

$$VO_{2max} = Q_{max} \times (Ca\text{-}vO_{2max}) = (SV_{max} \times HR_{max}) \times (Ca\text{-}vO_{2max}) \quad .$$

Q_{max} (or $SV_{max} \times HR_{max}$) reflects ”supply side” factors: the ability of the myocardium to increase stroke volume and the ability of the heart rate to increase (Figure 7). Ca-vO_{2max} reflects “demand side” factors: the ability of the muscle to extract oxygen from the blood and the fraction of blood circulating through metabolically active muscle (however, blood hemoglobin content, perhaps better seen as a supply side factor, also affects Ca-vO_{2max}). Thus, differences in VO_{2max} between people or changes in VO_{2max} (e.g. due to endurance training) must reflect a change in either Q_{max} or Ca-vO_{2max}.

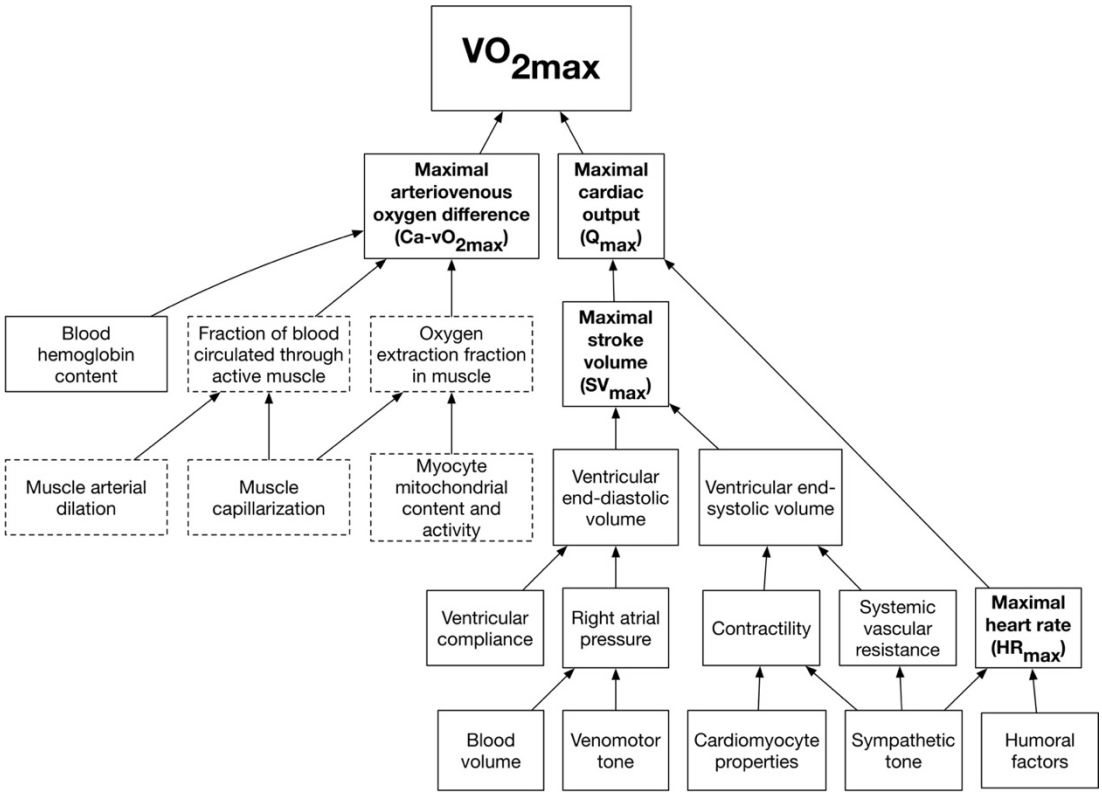

Figure 7 Illustration of the physiological factors determining VO_{2max}. Based on Boron & Boulpaep[194] and Hall[195]. Dashed boxes depict factors related to peripheral muscle tissue properties, solid boxes depict other factors.

Differences in VO_{2max} between healthy individuals are probably mainly explained by differences in Q_{max}, not Ca-vO_{2max}, as reviewed by Saltin & Calbet[196] and Levine[197]. Although in elite athletes the maximal arteriovenous oxygen difference (Ca-vO_{2max}) can be slightly higher than in non-athletes[198–200], the difference even then is small and does not account for their large relative VO_{2max} in these highly trained individuals[197]. The main difference between non-athletes and elite athletes in VO_{2max} seems to be explained by their capacity for increasing SV_{max} through having relatively more compliant left ventricles and higher contractility[197]. Thus, differences between healthy individuals in VO_{2max} are explained by differences in maximal cardiac output (Q_{max}), not increased Ca-vO_{2max}.

Furthermore, considering the effects of endurance training or exercise, two recent meta-analyses have shown that in studies of individuals with a mean age of less than 40 years[201] and individuals with a mean age of more than 40 years[193], the standardized mean differences in VO_{2max} and Q_{max} due to endurance training are significantly positively associated, whereas the standardized mean difference in Ca-vO_{2max} is not significantly associated with the change in VO_{2max}. In the meta-analysis of the younger individuals, the maximal heart rate (HR_{max}) was slightly lowered[201], whereas there was no significant differences in HR_{max} in the meta-analysis of the older

individuals[193], and thus, the increase in Q_{max} due to endurance training (explaining the increase in VO_{2max}) came from increased stroke volume (SV_{max}), not HR_{max}. To summarize, in healthy individuals, differences in VO_{2max} between individuals and the increase in VO_{2max} due to endurance training are explained by central cardiovascular factors determining maximal cardiac output (Q_{max}), not peripheral factors affecting oxygen extraction ($Ca\text{-}vO_{2max}$) (depicted in Figure 7 with dashed boxes). Some potential factors behind differences in Q_{max} are differences in left ventricular structure or function, peripheral vascular resistance, and blood volume[193]. From a theoretical viewpoint, then, it is not apparent that VO_{2max} (reflecting maximal cardiac output and thus primarily cardiovascular function) should be connected to insulin sensitivity (thought to reflect skeletal muscle function), lipids (thought to reflect liver and adipose tissue function), or resting blood pressure, making the possible association between VO_{2max} and metabolic health not seem straightforward.

In summary, cardiorespiratory fitness is usually defined as the maximal oxygen uptake (VO_{2max}) during exercise. VO_{2max} is the product of maximal cardiac output (Q_{max}) and the maximal arteriovenous oxygen difference ($Ca\text{-}vO_{2max}$). However, differences in VO_{2max} between individuals or changes in VO_{2max} due to endurance training seem to follow from differences or changes in maximal cardiac output (or maximal stroke volume), rather than from differences or changes in $Ca\text{-}vO_{2max}$. As maximal cardiac output mainly reflects central cardiovascular factors not directly connected to peripheral tissue function (Figure 7), in theory it would hardly be surprising if VO_{2max} was not strongly associated with metabolic health. This is because differences in VO_{2max} do not necessarily entail differences in the function of peripheral tissues that determine metabolism (e.g. muscle, liver, or adipose tissue).

2.4.2 MEASURING FITNESS INDEPENDENTLY OF ADIPOSITY

Next, I will review some issues regarding the measurement of CRF independently of obesity. Absolute VO_{2max} measures the maximal volume of oxygen consumption by tissues (mainly by skeletal muscle). Therefore, larger individuals will have higher VO_{2max} than smaller individuals due to their larger tissue mass, even with equal cardiorespiratory function. Thus, VO_{2max} must be scaled to body size, which presents some methodological difficulties. To enable comparisons of VO_{2max} between individuals there are generally two possible approaches: 1) to develop sex-, age-, and/or weight-specific reference values for VO_{2max} that correspond to the fitness levels, and assign people their level of fitness using these categories, and 2) to scale VO_{2max} to body size by adjusting it with a body size variable such as weight or fat-free mass (FFM) (or use regression-based methods to adjust for body size). Method 1 is not optimal for research purposes, as adjusting for a variable by grouping individuals

according to his/her values of that variable inevitably leads to residual confounding[202–205], thus not truly controlling for body size.

Regarding method 2, scaling VO_{2max} by dividing it by weight has generally been used widely in research[206] and is recommended in some clinical guidelines[192,207]. Scaling VO_{2max} by dividing it by weight has, however, at least two different kinds of problems: 1) controlling for the effect of a variable by dividing it with another requires that their relationship is linear with a zero intercept[208–211], and it has long been recognized, at least as early as in 1949 by Tanner[212], that for VO_{2max} and weight this does not apply[211–215] and 2) even if weight is used to scale VO_{2max} with methods that avoid the former problem, as body fat contributes significantly to weight, the scaling of VO_{2max} with weight confounds the measure of CRF with adiposity.

Multiple studies demonstrate that dividing VO_{2max} by weight does not properly control for the effect of weight on VO_{2max}[212,214–217], underestimating the CRF (as defined) of heavy or obese individuals[211,216,217], and it has been shown to produce spurious correlations between CRF and traits related to adiposity such as abnormal glucose metabolism[215], insulin sensitivity[218], and metabolic syndrome risk[218]. These studies indicate the VO_{2max}/weight standard should not be used and its use can lead to erroneous conclusions about the association of CRF and metabolic health.

Next, I will briefly review some theory on proper scaling of variables known to vary by body size. To enable the comparison of any trait that varies with body size between different individuals, the trait must be scaled by some body size measurement to make the measure of the trait comparable between individuals with different body sizes. A common example of this is BMI, where body weight is scaled by dividing it with the square of height to make it a measure of adiposity independent of body size (height). If one would use weight as a measure of adiposity, taller or larger individuals would be treated as adipose, whereas short and small individuals would be treated as lean. Similarly, VO_{2max} is associated with body size, so to make it comparable between individuals of different sizes, it must be scaled by some body size measure. The commonly used method of dividing VO_{2max} with body weight (VO_{2max}/weight) is problematic for the reasons reviewed above.

According to theory on allometric scaling, the proper way of scaling trait Y to body size measure X is by determining the scaling exponent of X with the following allometric model: $Y = aX^b\epsilon$, where a is a constant, b is the scaling exponent, and ϵ is the error term[211]. After the fitting the model to obtain the scaling exponent b, it can be used to scale individual values Y by individual values of X with the equation $Y_{allometric} = YX^{-b} = \frac{Y}{X^b}$, instead of the much used problematic ratio method ($Y_{ratio} = YX^{-1} = \frac{Y}{X}$), which is a special case of the allometric method with a scaling exponent b of 1[211].

There is no good basis, *a priori*, for expecting the scaling exponent to be exactly 1 for different pairs traits X and Y, and thus, the ratio method should not be used as a default without further consideration[211]. The logic between

choosing a scaling exponent different from 1 is the same as behind scaling body weight by the square of height (a scaling exponent of 2) in BMI, not just dividing weight by height. The proper scaling exponent *b* for scaling VO_{2max} by weight seems to be around 0.67[206,213,219], so if weight is used to scale VO_{2max}, it should be done with the equation $YX^{-0.67}$ (VO_{2max}/weight$^{0.67}$) to properly adjust for weight. While using VO_{2max}/weight$^{0.67}$ as a measure of CRF is an improvement to using VO_{2max}/weight, it is important to note that it does not fully control for the effects of adiposity since VO_{2max}/weight$^{0.67}$ is still associated with adiposity, as illustrated in Figure 23.

Instead of using weight, many authors recommend scaling VO_{2max} by fat-free mass (FFM) both for basic research[211,213,216,220] and for clinical physiology[221–223]. At least three kinds of arguments support using FFM instead of weight. 1) There is no theoretical reason to expect that fat mass itself affects VO_{2max} since only a minor amount of cardiac output is directed to adipose tissue in exercise[224]. 2) Fat mass (which is included in weight) is not correlated with VO_{2max}[225,226], whereas FFM is highly correlated with VO_{2max}[206,213,216,220,226]. 3) In clinical practice in the assessment of dyspnea and heart failure, the VO_{2max}/FFM standard has superior prognostic value[221,223]. Additionally, since the relationship between VO_{2max} and FFM is approximately linear with a zero intercept[213,216], a scaling exponent of 1 can be used, which is equal to dividing VO_{2max} by FFM, making the scaling process simpler. A comparison of the different methods for scaling VO_{2max} can be seen in Table 2.

Although there might not be any conclusive reasons to scale VO_{2max} by FFM instead of by weight$^{0.67}$, for research purposes, when comparing CRF and adiposity as predictors of metabolic health, the VO_{2max}/FFM standard for scaling has the advantage of being independent (or more independent) of adiposity than the VO_{2max}/weight$^{0.67}$ standard. Thus, if the effects of CRF and adiposity on metabolic health are examined jointly, using the VO_{2max}/FFM standard makes it possible to better separate the effects of CRF and adiposity on metabolic health, answering the question of whether adding a measure of VO_{2max} provides any explanatory power above and beyond what is provided by adiposity. If the VO_{2max}/weight$^{0.67}$ standard is used, as it is more correlated with adiposity, the variation accounted for by adiposity is to some extent included in the measure of CRF, and thus, does not directly reflect the additional value of measuring VO_{2max}.

Table 2. *Summary of different methods for scaling VO_{2max}.*

	VO_{2max} scaling method				
Method characteristics	**Unscaled**	**Age, sex, weight, and/or fat % specific fitness categories**	**Scaling by weight**	**Scaling by weight$^{0.67}$**	**Scaling by fat-free mass**
Ratio assumption violated[a]			Yes	No	No
Increases residual confounding[b]		Yes	No	No	No
Underestimates CRF in small subjects	Yes	Yes	No	No	No
Underestimates CRF in obese subjects	Yes	Yes	Yes	Yes	No
How common in research[c]	Very uncommon	Common	Common	Very uncommon	Uncommon

[a]Controlling for the effect of variable X on Y by dividing Y with X requires that their relationship is linear with a zero intercept[208–211]. [b]Adjusting variable Y with X by grouping individuals according to their values of X leads to residual confounding by X[202–205]. [c]Based on the view of the author, no systematic review was performed.

In addition to the problems with scaling VO_{2max}, there are possible problems associated with estimating VO_{2max} with exercise tests when a direct measure of VO_{2max} is not available. Even if VO_{2max} values were to be scaled properly, if VO_{2max} is estimated indirectly from performance weight-bearing exercise tests (e.g. treadmill tests), this can still bias the VO_{2max} estimate against obese individuals since fat mass contributes to a higher workload during the test[227]. Common procedures used to estimate VO_{2max} from performance in a treadmill exercise test include the Balke protocol[228] and the Bruce protocol[229]; they estimate VO_{2max} from the duration of exercise sustained with increasing workload. However, these exercise test are validated against direct VO_{2max} measurements in normal-weight subjects only[227,230]. Zhu et al.[231] studied how body composition affects performance in a treadmill fitness test used to estimate VO_{2max} (modified Balke protocol). The duration of treadmill exercise sustained was lowered by 7.5 seconds per kilogram of fat mass, after controlling for FFM, height, and other factors[231]. The standard deviation for exercise duration was approximately 135 seconds for men and women combined[231], thus ~18 kilograms of additionall fat mass alone would make the subject be classified one standard deviation lower in CRF. This demonstrates that VO_{2max} estimated from weight-bearing exercise tests is biased against more obese individuals and leads to the estimate of VO_{2max} being negatively correlated with adiposity[227], which leads to spurious relationships between the biased VO_{2max} estimate and variables associated with adiposity. Without properly controlling adiposity, e.g. by regression-based methods, VO_{2max} estimated from weight-bearing exercise is not a measure of CRF independent

of adiposity. This is especially a problem with multiple large-scale prospective epidemiological studies on the effects of CRF on mortality[15,232–240]. Cycle ergometry tests without direct VO_{2max} measurement, such as the protocol used in the Danish subjects in Study III[241], are probably less biased against obese subjects since in theory the workload in these tests does not heavily depend on fat mass since only the weight of the legs can increase the workload because other parts of the body are supported by the seat.

Table 3. *Summary of different methods for VO_{2max} measurement or estimation.*

	Weight-bearing exercise		Non-weight-bearing exercise	
VO_{2max} method	**VO_{2max} estimated from time of test sustained**	**VO_{2max} measured directly**	**VO_{2max} estimated from maximal workload**	**VO_{2max} measured directly**
Underestimates fitness in obese	Yes	No	No[a]	No
How common in studies[b]	Common[c]	Uncommon	Common	Common (small studies)
In this thesis			Study III Danish sample	Study III Finnish sample

[a]Probably not at least to the same extent as in weight-bearing exercise tests; although the different methods for estimating VO_2 from workload are validated against direct VO_2 measurements[228,229,241,242], the methods used have not to my knowledge been validated in obese subjects separately. [b]Based on the view of the author, no systematic review was performed. [c]Common, especially in large epidemiological cohorts[15].

In summary, scaling VO_{2max} to body size by dividing it with weight (VO_{2max}/weight) is a problematic method, confounding the resulting measure with adiposity (Table 2). A better way to scale VO_{2max} seems to be to divide it with fat-free mass (VO_{2max}/FFM). Furthermore, if VO_{2max} is estimated indirectly from performance in weight-bearing exercise tests, the estimate of VO_{2max} is biased against obese subjects due to the increased workload during the exercise test, which is yet another way that measures of CRF can be confounded by adiposity (Table 3). These problems with measuring or scaling VO_{2max} independently of adiposity lead to difficulties in interpreting the results of studies examining the association between CRF and metabolic health.

2.4.3 CARDIORESPIRATORY FITNESS AND INSULIN SENSITIVITY

There are multiple studies on humans regarding the relationship between CRF and insulin sensitivity, metabolic syndrome risk and its subcomponents, ectopic fat accumulation, and mortality or cardiovascular disease outcomes. First, we will review the studies regarding insulin sensitivity that have measured VO_{2max} in ways probably not confounded with adiposity. Although VO_{2max}/weight has been shown to be associated with insulin sensitivity or glucose tolerance in many studies (e.g.[243,244]), I will refrain from reviewing these and other similar studies since they have problems in separating the

effects of adiposity from those of CRF because the CRF measure is confounded by adiposity for the reasons reviewed above.

Sævarsson et al.[245] studied 127 subjects aged 17-23 years with BMIs of around 24 kg/m^2. VO_{2max} was estimated indirectly with maximal bicycle ergometry and divided by FFM. They observed that after adjusting for age and sex, VO_{2max}/FFM was significantly negatively correlated with HOMA-IR (r = −0.29), but after additionally adjusting for fat %, the partial correlations were not significant.

Huth et al.[246] studied 53 men sampled in four different groups: sedentary controls without obesity (BMI < 25 kg/m^2), sedentary with obesity (BMI > 30 kg/m^2), sedentary with obesity and glucose intolerance, and endurance-trained active without obesity. VO_{2max} was directly measured with a maximal spiroergometry test and divided by FFM. VO_{2max}/FFM correlated very highly with insulin sensitivity (from a hyperinsulinemic-euglycemic clamp) (r = 0.78, or r = 0.56 with the endurance-trained group excluded). Adding VO_{2max}/FFM to a regression model with waist circumference and adiponectin, VO_{2max}/FFM increased the R^2 by 0.08, which corresponds to a semipartial correlation of r = 0.28. However, despite the gold standard measures of CRF and insulin sensitivity, these results warrant skepticism due to the fact that the correlations were calculated across heterogeneous groups (there were highly significant differences between the different groups in VO_{2max}/FFM and insulin sensitivity), since when calculating a Pearson's correlation coefficient the variables should be normally distributed (or more precisely, their bivariate joint distribution should be normally distributed [247]), and here this is probably not the case. This might inflate the estimated correlation coefficients. Unfortunately, the authors did not provide plots of the associations.

McMurray et al.[218] studied 1784 subjects aged 8-18 years. They indirectly estimated VO_{2max} from a maximal bicycle ergometry test. VO_{2max}/FFM was significantly correlated with HOMA-IR (r = -0.11), however, after adjusting for sex, ancestry, height, and fat %, the association was not significant (r = -0.03).

Henderson et al.[248] studied 630 children aged 8-10 years. VO_{2max} was measured directly with a maximal spiroergometry test and divided by FFM. VO_{2max}/FFM was not significantly associated with insulin sensitivity (HOMA-IR or Matsuda index), whereas adiposity and moderate to vigorous physical activity were significant predictors. In contrast, VO_{2max}/FFM was significantly associated with second-phase insulin secretion (insulin secretion measured by the ratio of the area under the curve of insulin to the area under the curve of glucose during the full 2 hours of the oral glucose tolerance test), although the authors did not provide standardized effect sizes.

Morinder et al.[249] studied 228 children aged 8-16 years, who were predominantly severely obese (mean BMI 35.5 kg/m^2). VO_{2max} was estimated indirectly with a submaximal bicycle ergometry test and divided by FFM. Insulin sensitivity (FSIVGTT) was significantly correlated with VO_{2max}/FFM (r = 0.36). Additionally, in a multiple linear regression model with sex, BMI SDS, age, VO_{2max}/FFM, Tanner stage and fat % as predictors, VO_{2max}/FFM was

associated with an additional R^2 or 0.06, corresponding to a semipartial r of 0.24. These results are in contrast with Henderson et al.[248], who did not find a significant association in children aged 8-10 years. However, it is possible that in Morinder et al.[249] the estimated VO_{2max} is to some extent confounded by adiposity since to my knowledge the submaximal ergometry protocol has been validated for normal-weight adults[242], not for obese children.

In summary, the reviewed heterogeneous studies estimating the association of VO_{2max}/FFM with insulin sensitivity or resistance give somewhat conflicting results, with estimates ranging between $|r|$ = 0.11 and 0.78. However, the two high-quality studies with large sample sizes and direct VO_{2max} measurement were performed on children or youth, and these studies give more modest estimates: McMurray et al.[218] examining 1784 subjects aged 8-18 years with $|r|$ = 0.11, and Henderson et al.[248] examining 630 children aged 8-10 years with no significant association. Thus, the high-quality studies on children and youth show no meaningful association between VO_{2max}/FFM and insulin sensitivity. We did not find any high-quality studies on older individuals, thus, high-quality studies on adults examining the association between VO_{2max}/FFM and insulin sensitivity appear to be lacking.

Table 4. *Summary of reviewed studies on the association between VO_{2max}/FFM and insulin sensitivity.*

Study	Subjects	Age	VO_{2max} measure	Result	Note
Sævarsson et al.[245]	N = 127, BMI ~ 24 kg/m^2	17-23	Bicycle ergometry, scaled with FFM	VO_{2max}/FFM significantly correlated with HOMA-IR, r = −0.29	Adjusting for fat %, no significant association
Huth et al.[246]	N = 53, BMI 24-35 kg/m^2, four groups heterogeneous for BMI/CRF	~44	Bicycle ergometry, scaled with FFM	VO_{2max}/FFM correlated with insulin sensitivity r = 0.78	After adjusting, semipartial r = 0.28
McMurray et al.[218]	N = 1784, BMI ~ 22 kg/m^2	8-18	Bicycle ergometry, scaled with FFM	VO_{2max}/FFM was correlated with HOMA-IR, r = -0.11	After adjusting for sex, ancestry, height, and fat %, r = -0.03
Henderson et al.[248]	N = 630, 56% normal weight, 19% overweight, 23% obese	8-10	Spiro-ergometry, scaled with FFM	VO_{2max}/FFM not associated with HOMA-IR or Matsuda index	
Morinder et al.[249]	N = 228, BMI ~ 36 kg/m^2	8-16	Bicycle ergometry, scaled with FFM	VO_{2max}/FFM correlated with insulin sensitivity, r = 0.36	Adjusting for other variables, semipartial r = 0.24

Additionally, at least one study[250] has tried to control for the effects of adiposity in the association between VO_{2max}/weight and outcome variables by multivariate statistics. Solomon et al.[250] studied 313 subjects sampled in three heterogeneous groups: 137 normal glucose tolerance subjects with mean BMI of 27 kg/m^2, 85 impaired glucose tolerance subjects with mean BMI of 33

kg/m², and 91 subjects with T2DM with mean BMI of 31 kg/m². They measured VO_{2max} directly in a maximal exercise test and divided it by weight. Insulin sensitivity was estimated with an OGTT. There were highly significant differences between some of the groups in VO_{2max}/weight, HbA1c, fasting glucose, and insulin sensitivity. The authors show significant correlations between VO_{2max}/weight and HbA1c (r =-0.33), fasting glucose (r = -0.34), and insulin sensitivity (r = 0.73) in all subjects, although the associations with HbA1c and fasting glucose are not significant among the subgroup of subjects with T2DM. But from these associations it is not possible to say whether they are confounded by adiposity since the authors used VO_{2max}/weight. They did, however, provide a few multiple regression models, where they included VO_{2max}/weight, age, sex, weight, BMI, and fat % as the predictors. When predicting HbA1c (R^2 = 0.20), fasting glucose (R^2 = 0.15), and insulin sensitivity (R^2 = 0.86), VO_{2max}/weight has significant βs of -0.83, -0.68, and 0.34, respectively. The βs from these models are hard to interpret since in a regression model with a correlated set of predictors (VO_{2max}/weight, weight, BMI, and fat %), the estimated βs can be very large due to their intercorrelations and correlations with the outcome variable, whereas the change in R^2 from leaving one of the predictors out can be arbitrarily small. So β coefficients do not correspond well with the unique variance explained by any given variable if multiple predictors are correlated with one another[251]. Unfortunately, Solomon et al.[250] do not provide semipartial correlations or the change in R^2 if VO_{2max}/weight were omitted, which would allow the assessment of unique variance explained in the outcome variables by VO_{2max}/weight, apart from that explained by the adiposity variables. Thus, it is not possible to determine from Solomon et al.[250] the unique contribution of VO_{2max}/weight to abnormal glucose metabolism and insulin sensitivity.

2.4.4 CARDIORESPIRATORY FITNESS AND METABOLIC SYNDROME

Metabolic syndrome is characterized by obesity or abdominal obesity (high waist circumference), elevated fasting glucose, hypertriglyceridemia, low HDL levels, and hypertension[36]. For research purposes, the combined effect of these subcomponents is sometimes used to form a continuous metabolic syndrome score. While some factor analysis-based techniques for calculating the score exist (e.g. Viitasalo et al.[252]), the subcomponents are commonly just standardized to have a mean of 0 and standard deviation of 1 (by Z-scoring), and these Z-scored variables are added together to obtain a score that depicts metabolic syndrome in a continuous manner[253–257].

There are some cross-sectional studies that show an association between VO_{2max}/weight and a metabolic syndrome score[257] or odds of metabolic syndrome diagnosis[258,259]. It is, however, unclear to what extent these associations are confounded by adiposity. However, in Sævarsson et al.[245], VO_{2max}/FFM is significantly associated with total cholesterol (r = -0.20) and triglycerides (r = -0.25), and in McMurray et al.[218] VO_{2max}/FFM is significantly

associated with mean blood pressure (r = -0.18) and a metabolic syndrome score (r = -0.13), but not with total cholesterol, HDL cholesterol, or triglycerides. Longitudinal studies might, however, be more suited to assessing the relationship between CRF and metabolic syndrome; a change in a CRF measure may possibly be less confounded by adiposity since the same individual is studied at multiple time points. Gibbs et al.[260] studied 4408 adults with T2DM undergoing either an intensive lifestyle intervention or diabetes support and education. They assessed CRF with a maximal treadmill exercise test at baseline and at a one-year follow-up. In regression models including age, race, gender, change in medication, and baseline value of the outcome variable as covariates, they reported the R^2s of the models with the covariates and weight change, and models with CRF change added as an additional predictor. The increases in model R^2 (ΔR^2) due to adding CRF change as a predictor (after weight change was added) were for systolic blood pressure ΔR^2 = 0.000, diastolic blood pressure ΔR^2 = 0.000, fasting glucose ΔR^2 = 0.007, HbA1c ΔR^2 = 0.011, LDL cholesterol ΔR^2 = 0.001, HDL cholesterol ΔR^2 = 0.004, and triglycerides ΔR^2 = 0.002, or expressed as semi-partial correlations (taking the square root of the ΔR^2): r = 0.00 to 0.10[260]. Lee et al.[261] studied 3148 healthy adults over a 6-year follow-up. They estimated CRF from performance on a maximal treadmill test. They report the following partial correlations between CRF change and change in metabolic health measures, adjusted for age, sex and change in fat %: systolic blood pressure r = -0.05, diastolic blood pressure r = -0.04, fasting glucose r = 0.03 (not significant), triglycerides r = -0.10, HDL cholesterol r = 0.08, and total cholesterol r = -0.05. Thus, in these two large longitudinal studies, change in CRF did not account for any meaningful amount of variance in the change of the metabolic health variables above that explained by the covariates and weight or fat % change. In summary, the reviewed cross-sectional and longitudinal studies, measuring CRF as VO_{2max}/FFM or controlling for body composition, show weak or no associations between CRF and metabolic health variables associated with metabolic syndrome.

Ectopic fat accumulation (visceral or liver fat) has been suggested to possibly mediate the relationship between CRF and metabolic health[262–264]. Arsenault et al.[262] studied 169 men without T2DM, measuring CRF as VO_{2max}/weight. They show that when the subjects are divided into tertiles of VO_{2max}/weight, men in the lowest VO_{2max}/weight tertile have more visceral fat than men in the highest VO_{2max}/weight tertile, even when matched for BMI. They interpret this to mean that visceral fat accumulation might be associated with CRF. However, even when matched for BMI, the men in the low VO_{2max}/weight group had less muscle mass and more fat mass than the high VO_{2max}/weight group[262]. Since VO_{2max}/weight is correlated with adiposity, the subgrouping of the subjects with equal BMI also leads to subjects in the low VO_{2max}/weight group being more adipose than subjects in the high VO_{2max}/weight group. Thus, the association between visceral fat accumulation and VO_{2max}/weight grouping in this study can be explained by differences in

adiposity in the groups, even without CRF influencing visceral fat accumulation. Haufe et al.[264] studied 138 adults with a mean BMI of 34 kg/m². Based on their observation that the correlation between VO_{2max}/weight and insulin sensitivity becomes smaller and non-significant after controlling for liver fat %, they suggest that the effects of CRF on insulin sensitivity might be mediated through liver fat content. However, their results might be interpreted as follows: if VO_{2max}/weight is associated with insulin sensitivity through its association with adiposity, controlling for liver fat % (which is correlated with adiposity) removes this association due to controlling for adiposity, thus, their result can be explained without an independent effect of CRF on ectopic liver fat accumulation.

Adiposity[265] and CRF[266] have both been separately shown to predict mortality in large meta-analyses. However, their joint associations with mortality are less clear. Barry et al.[15] published a meta-analysis examining the joint association of CRF and adiposity on mortality and argue based on their results that if you take CRF into account adiposity does not predict mortality. They show that normal-weight fit individuals, overweight fit individuals, and obese fit individuals all have approximately equal mortality. Conversely, unfit normal-weight individuals, unfit overweight individuals, and unfit obese individuals all have higher mortality than normal-weight fit individuals. Thus, if you stratify people into fit and unfit categories, the association between obesity and mortality disappears.[15]

While these results may at first glance seem to show that CRF is more important with respect to mortality than adiposity, upon closer inspection the interpretation of their results is not that straightforward. Nine out of ten of the studies in the meta-analysis estimated CRF with a weight-bearing treadmill exercise test, without direct VO_{2max} measurement[232,233,233–236,238,239], whereas the one study measuring VO_{2max} directly classified subjects into high and low fitness categories using a sex-specific median split of the study population[267]. As discussed earlier, estimating VO_{2max} or CRF from a weight-bearing exercise test penalizes those subjects with higher fat mass due to the higher workload[227]. Therefore, the nine studies actually have a CRF measure that is confounded by adiposity. And in the one study with direct VO_{2max} measurement[267], splitting absolute VO_{2max} values at the median causes more adipose individuals to be classified in the low CRF group because FFM is correlated with VO_{2max}[206,213,216,220,226], while fat mass is not[225,226]. Thus, the classification of subjects to fit and unfit groups in Barry et al.[15] is confounded by adiposity, with unfit individuals having more fat mass than fit subjects. As the fit and unfit groups are further stratified according to BMI, this leads to unfit individuals having more fat mass relative to fit individuals within each BMI category. Thus, the different mortality within normal fit, overweight, and obese groups might just as well be explained by differences in adiposity within these BMI groups. It thus might be impossible to determine from the data in these studies whether the association of CRF with mortality is truly independent of adiposity.

Furthermore, the studies included in Barry et al.[15] include subjects with mean ages of 44 to 64 years, and three studies included diabetics/prediabetics, one study included coronary artery disease patients, and one included hypertension patients. In older subjects CRF might be limited by cardiopulmonary disease, and thus the association between CRF and mortality demonstrated in Barry et al.[15] might not be driven by metabolic health per se, but by the increased mortality associated with cardiopulmonary disease. If cardiopulmonary disease, which leads to a reduction in CRF, is in part caused by obesity in the long-term, the results of the study do not undermine the importance of adiposity, even if in old age CRF is a stronger marker for cardiopulmonary disease.

In summary, the literature on the association between CRF and insulin resistance, metabolic syndrome, and mortality is problematic to interpret since most of the measures used for CRF are confounded with adiposity. However, the results from high-quality studies, either measuring CRF as VO_{2max}/FFM or properly adjusting for body composition using multivariate statistics, show no or very weak associations between CRF and insulin sensitivity[218,248] or metabolic syndrome components[245,260,261]. Additionally, I found no high-quality studies in adults that examined the associations of VO_{2max}/FFM with insulin sensitivity, metabolic syndrome components, or a continuous metabolic syndrome. In Study III, we aimed to address this lack of studies measuring the effect of CRF on metabolic health, independently of adiposity, in adults.

3 AIMS

The aims of Study I, investigating healthy adult MZ twin pairs, some discordant for BMI, were as follows:

- To assess the phenotypic associations of subcutaneous adipose tissue (SAT) *SIRT* expression with measures of adiposity and ectopic fat accumulation, measures of systemic insulin sensitivity and SAT insulin signaling gene expression, and measures of systemic and SAT inflammation.
- To assess the phenotypic associations of SAT NAD^+ synthesis gene expression with the same measures of adiposity, insulin action, and inflammation.
- To control for the possible confounding effects of genetic and shared environmental factors in the abovementioned associations by evaluating intrapair differences within MZ pairs.
- To determine whether acquired obesity is associated with differences in SAT UPR^{mt} pathway gene expression in MZ twin pairs discordant for BMI.

The aims of Study II, investigating obese subjects participating in a weight loss intervention, were as follows:

- To evaluate how SAT *SIRT*, *NAMPT*, and *PARP* expression and SAT total PARP activity are altered relative to lean controls and after long-term weight loss.

The aims of Study III, investigating two samples of healthy adult MZ and DZ twin pairs, were as follows:

- To examine the phenotypic associations of cardiorespiratory fitness (CRF, defined as VO_{2max}/FFM), adiposity, and fat-free mass (FFM) with measures of metabolic health (insulin sensitivity, fasting glucose, metabolic syndrome components, a metabolic syndrome risk score, visceral fat amount, and liver fat amount).
- To control for possible confounding effects of genetic and shared environmental factors in these associations by examining intrapair differences within MZ twin pairs.
- To determine whether variation in CRF, adiposity, or FFM best explains the variation in the measures of metabolic health.

4 MATERIALS AND METHODS

4.1 STUDY SUBJECTS

4.1.1 TWINFAT STUDY (STUDIES I, II, AND III)

The TwinFat sample is a cross-sectional mixed-sex sample drawn from two Finnish population-based longitudinal studies of five consecutive birth cohorts of twins: FinnTwin16 and FinnTwin12[268]. Participants for TwinFat were selected according to their BMIs at the age of 23-27 years to include a wide range of intrapair differences in BMI. Therefore, a part of the TwinFat sample was not randomly selected; the sample is enriched with 20 MZ and 53 DZ pairs discordant for BMI (ΔBMI > 3 kg/m^2) and 18 MZ and 13 DZ pairs concordant for BMI (ΔBMI < 1 kg/m^2), but otherwise it was a random sample of the pairs (Figure 8). Exclusion criteria for the study were pregnancy or breastfeeding, abuse of alcohol or drugs, and diagnosis of diabetes or heart disease. However, one heavier co-twin of the BMI-discordant pairs had T2DM (with metformin and insulin as treatment) and another had inactive ulcerative colitis (treated with mesalazine and azathioprine). The twins were otherwise healthy. The ethics committees of the hospital districts of Southwest Finland and Helsinki and Uusimaa approved the study protocol, and the study was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all subjects.

Two different subsamples of TwinFat were included in Studies I and III. The sample for Study I included 26 MZ pairs discordant for BMI (ΔBMI > 3 kg/m^2) and 14 MZ pairs not discordant for BMI (ΔBMI < 3 kg/m^2); all subjects went through more comprehensive phenotyping, with SAT transcriptomics analyses by Affymetrix U133 Plus 2.0 chips. The twins were aged 22-36 (median 32) years. The TwinFat subsample used in Study III consisted of all MZ or DZ twin pairs from TwinFat with measured VO_{2max}. There were 153 such pairs aged 23-32 (median 28) years at the time of examination; 74 were MZ and 79 same-sex DZ pairs. See Figure 8 for an illustration of the sampling procedure of TwinFat subjects for Studies I, II, and III.

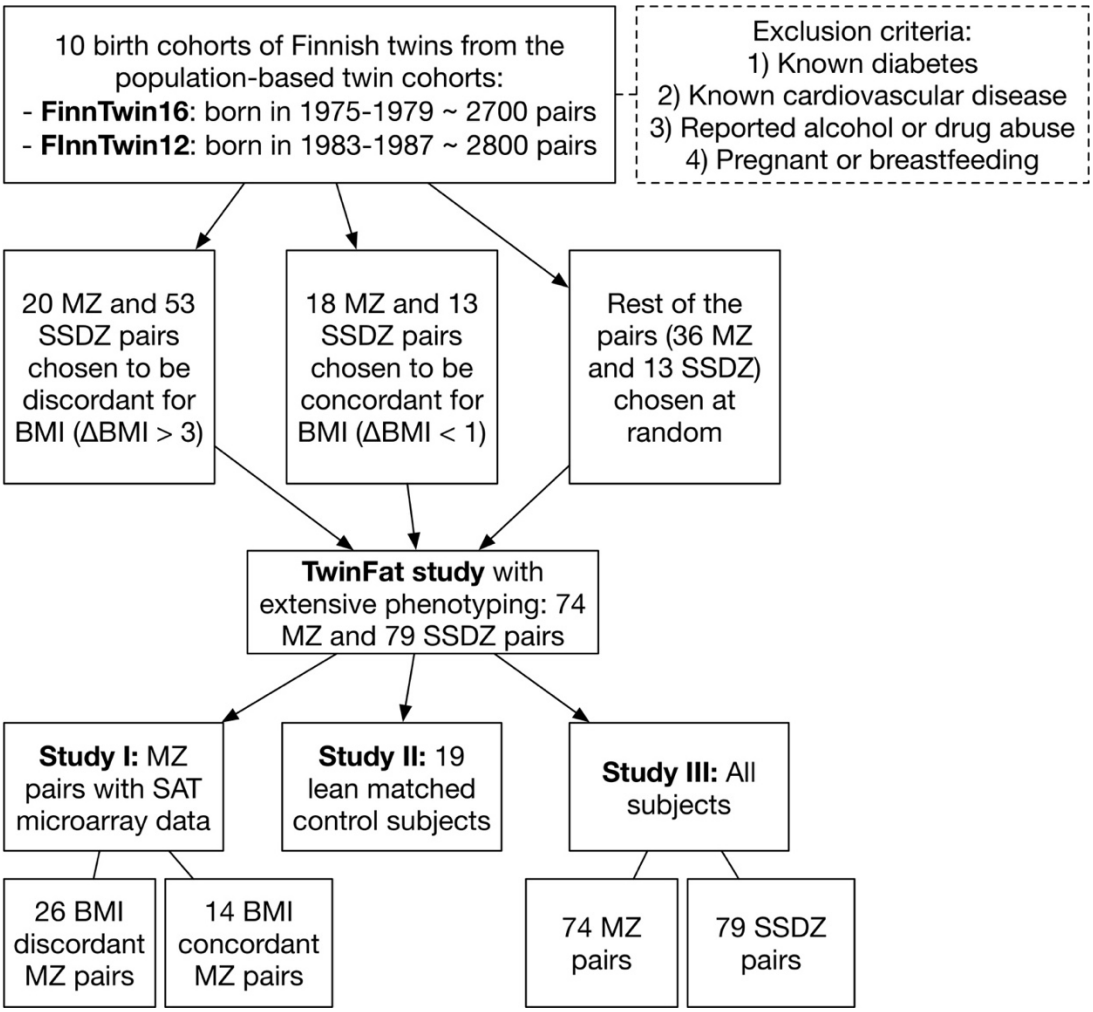

Figure 8 Sampling procedure for the Finnish twins for the TwinFat study (i.e. participants for Studies I, II, and III).

4.1.2 GEMINAKAR STUDY (STUDY III)

The twins for GEMINAKAR were recruited from two cohorts of the nationwide population-based Danish Twin Registry[269]. Cohort I covers the birth cohorts 1931–1952, while cohort II covers birth cohorts 1953–1982. Altogether 2585 randomly chosen pairs fulfilling the inclusion criteria from the cohorts were sent invitations to take part in a full-day clinical investigation (Figure 9). Cohort II was furthermore chosen based on a previous self-report of being healthy. The invitation letter had detailed information about the study and its exclusion criteria (e.g. known diabetes or cardiovascular disease, conditions precluding a progressive maximal bicycle test, pregnancy, and breast-feeding). A reply coupon was enclosed for the twins to reply with information about their present health status and whether they would agree to a telephone contact. If one twin of the pair did not respond or did not want to participate in the study, the pair was excluded. Twins were additionally excluded at recruitment if they were pregnant or breastfeeding, reported abuse of alcohol

or drugs, or had been diagnosed with diabetes or heart disease. Altogether 1098 complete twin pairs (42.5%) were both willing and able to participate in the study. A stratified sample of 756 twin pairs underwent an extensive full-day clinical examination of a variety of phenotypes. Of these pairs, only 459 (60.7%) were included in the current study because the remaining pairs had missing body composition data due to equipment failure with the bioelectrical impedance device. Pairs missing because of this were essentially missing at random. The current sample from GEMINAKAR consisted of 459 twin pairs aged 18-67 (median 38) years at the time of examination. There were 182 MZ, 179 same-sex DZ, and 98 opposite-sex DZ pairs. The GEMINAKAR study was approved by all of the Danish regional scientific ethical committees and the Danish Data Protection Agency and was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

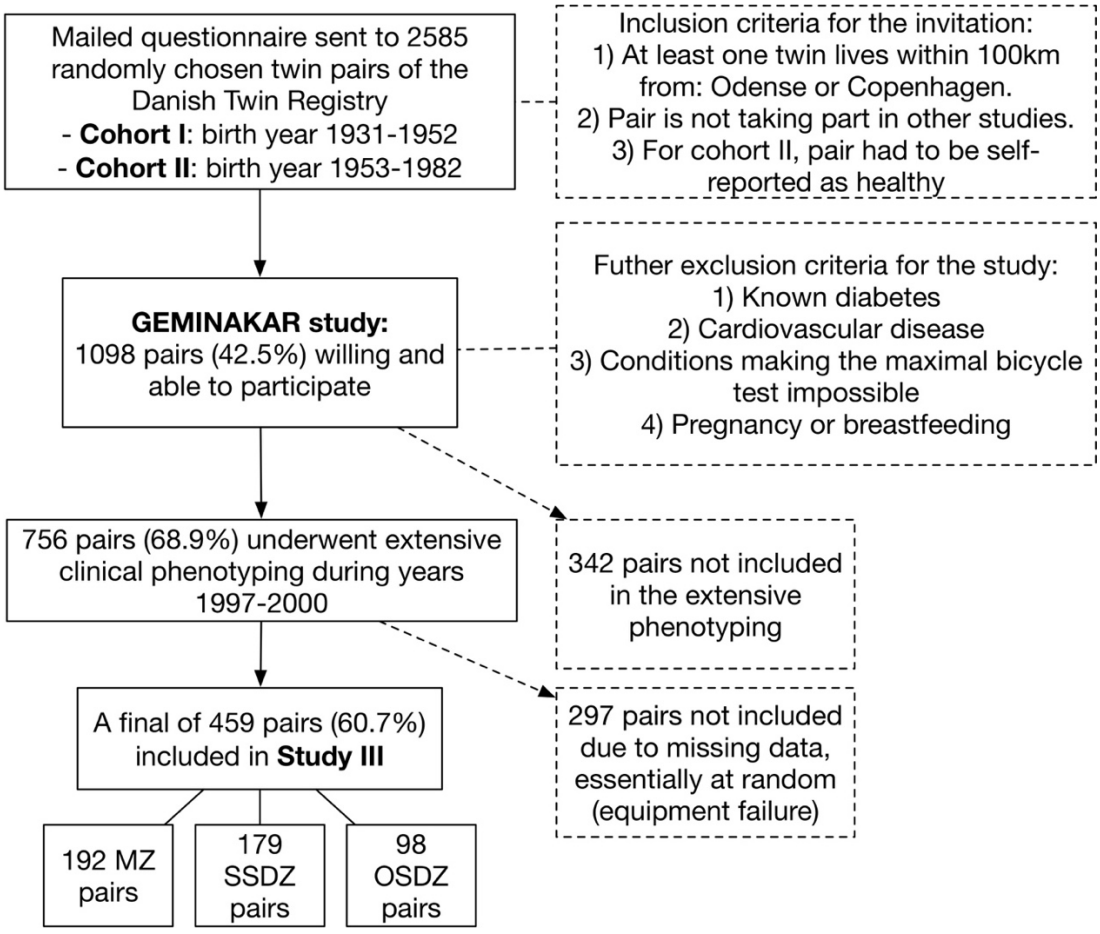

Figure 9 Sampling procedure for the Danish twins for the GEMINAKAR study (i.e. participants for Study III).

4.1.3 WEIGHT LOSS STUDY (STUDY II)

Nineteen obese (BMI 30-39, median 34 [kg/m^2]) volunteers were recruited for a 12-month weight loss program. The subjects were recruited with newspaper advertisements and consisted of healthy obese weight-stable subjects. Exclusion criteria were smoking, weight change of >5 kg in the last 3 months, diabetes, endocrinological disease, or drug treatment affecting food intake or weight regulation. Subjects were aged 20-48 (median 37) years, and 7 were male and 12 female. Subjects were assessed at baseline, 5 months, and 12 months. The ethics committees of the hospital districts of Southwest Finland and Helsinki and Uusimaa approved the study protocol, and the study was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all subjects. Nineteen sex- and age-matched lean reference subjects were chosen from the sample of Study I and were assessed similarly, apart from imaging studies (see Figure 10 for the examination protocol).

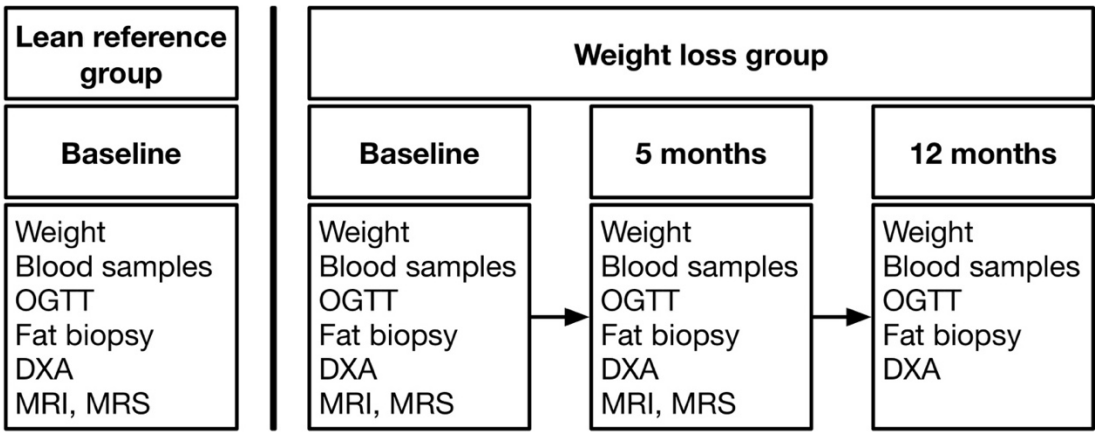

Figure 10 Examination protocol for the weight loss study (Study II). DXA, dual-energy x-ray absorptiometry; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; OGTT, oral glucose tolerance test. Adapted from Rappou et al.[270].

The weight loss intervention for the 19 obese subjects consisted of group-based counselling on exercise. The first 6 weeks of the intervention involved a very-low-energy diet (VLED) of 800-1000 kcal/day, after which the subjects were instructed to continue with a 500-1000 kcal/day restriction from their baseline recommended diet. VLED products for the 6 weeks were provided by Nutrilett and Allevo. The same nutritionist gave all the instructions. Subjects were instructed to eat at least 0.5 kg of vegetables and high-protein snacks with a target of 70-90 g of daily protein intake during the VLED intervention. During the whole intervention, after the VLED phase, a total daily intake of protein of 1.2-1.5 g/kg was instructed. Subjects were additionally provided with multi-vitamins (Multi-Tabs, Orion Pharma). Physical activity was recommended according to the national Finnish guidelines on obesity and

weight loss of 2011[271]. All subjects attended counselling sessions on lifestyle twice a month for 5 months, after which the sessions were continued once a month until the end of the 12-month follow-up. Diet counselling sessions for individuals were held at 0, 2, and 5 months.

4.2 MEASURES

4.2.1 BODY COMPOSITION AND CLINICAL ASSESSMENT

For all studies, weight was measured in light clothing with a beam scale (GEMINAKAR) or an electronic scale (TwinFat and weight loss study participants), and waist circumference was measured midway between the lowest rib margin and the anterior superior iliac spine. All measurements were made by trained examiners. Body composition was analyzed by dual-energy x-ray absorptiometry (DEXA, Prodigy, Lunar Corp., GE, Madison, Wisconsin, software version 2.15) in TwinFat and the weight loss study, and by bioelectrical impedance analysis (103 RJL- System analyzer, RJL-Systems, Detroit, MI, USA) in GEMINAKAR as detailed in Hasselbalch et al.[272]. BMI was calculated as weight/height^2, and for Study III fat mass index (FMI) was calculated as fat mass/height^2 and fat-free mass index (FFMI) was calculated as fat-free mass/height^2. Blood pressure was measured with mercury sphygmomanometers after rest (the mean of three subsequent measurements by trained examiners). For some of the subjects in TwinFat and all of the weight loss study participants, VAT amount and liver fat % were measured with magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS), respectively, as described elsewhere[273].

4.2.2 CARDIORESPIRATORY FITNESS

For Study III, participants in TwinFat and GEMINAKAR completed a maximal exercise test using a braked cycle ergomenter. In GEMINAKAR the test started with a 7-min warm-up at a submaximal starting workload of 103 W for men and 69 W for women, after which the workload was then increased by 35 W every 2 min for both sexes, until exhaustion. Maximal power output (MPO) was estimated as the power output before the last increase in workload plus the power output in the last workload multiplied by the percentage of time that the last power output was sustained from 2 min: MPO = (W_{max} - 35 W) + 35 W × (t_{max} / 120 s), where W_{max} is the maximal workload in Watts and t_{max} is the time that the maximal workload was sustained in seconds. The maximal power output was then used to estimate VO_{2max} with the formula derived and validated against direct VO_2 measurement by Andersen[241]: VO_{2max} = 0.0117 × MPO + 0.16, where VO_{2max} is expressed as L/min. Subjects in TwinFat completed a maximal exercise test using a Vmax spiroergometer (Sensorimedics, Yourba Linda, CA, USA). O_2 uptake and CO_2 production were

measured breath-by-breath. Exercise was continued to exhaustion, which was defined as a perceived exertion of 19 to 20 on the Borg scale or gas exchange ratio VCO_2/VO_2 of over 1.1. VO_{2max} was defined as the mean VO_2 observed during last 30 s of the test. For Study III, cardiorespiratory fitness was defined as VO_{2max} divided by fat-free mass (VO_{2max}/FFM), where FFM was estimated in TwinFat with DEXA and with fat mass from bioelectrical impedance and total body weight in GEMINAKAR.

4.2.3 LABORATORY MEASURES AND DERIVED INDICES

Fasting venous blood samples were drawn to measure fasting glucose, fasting insulin, total cholesterol, HDL cholesterol, and triglycerides in all studies. LDL was estimated using the Friedewald formula (LDL = total cholesterol - HDL cholesterol - triglycerides/5)[274]. Fasting plasma adiponectin was measured by an enzyme-linked immunosorbent assay. In TwinFat, additionally, serum hs-CRP, plasma adiponectin, and serum eNampt were measured. Details of the laboratory measurements (e.g. the specific assays used) can be seen in Benyamin et al.[275] for GEMINAKAR, Jukarainen et al.[276] for TwinFat, and Rappou et al.[270] for the weight loss study.

Subjects in TwinFat and GEMINAKAR underwent standardized 75 g oral glucose tolerance tests (OGTT), where plasma glucose and insulin levels were measured at 0, 30, and 120 min. HOMA-IR, an insulin resistance index using fasting values of glucose and insulin, was calculated as HOMA-IR = fasting glucose × fasting insulin × 22.5. Also the OGTT values of glucose and insulin were used to calculate the Matsuda index (an insulin sensitivity index)[277], BIGTT-SI (an insulin sensitivity index)[278], and BIGTT-AIR (an acute insulin response index)[278].

In Study III, a continuous metabolic syndrome score was formed from the components of metabolic syndrome, as defined in the NCEP ATP III guidelines[36]. The score was calculated from $\log_e$-transformed or untransformed variables as follows: metabolic syndrome score = waist circumference + (systolic blood pressure + diastolic blood pressure)/2 - $\log_e$(HDL) + $\log_e$(triglycerides). While this exact way of calculating a continuous metabolic syndrome score has not to my knowledge been validated, see Viitasalo et al.[252] for a validation of a similar score.

4.2.4 GENE EXPRESSION ANALYSES

For Studies I and II, periumbilical subcutaneous adipose tissue biopsies were obtained under local lidocaine anesthesia by a surgical technique and snap-frozen in liquid nitrogen. A part of the biopsy was not frozen and instead treated with collagenase to separate the adipocytes from the stroma vascular fraction for measurement of adipocyte size under a light microscope, as detailed in Heinonen et al.[24]. Total RNA was extracted from the frozen SAT biopsies as described in Heinonen et al.[22]. Transcriptomics analyses were

performed with the Affymetrix U133 Plus 2.0 microarray, and the expression of some of the genes was validated against quantitative RT-PCR as described in Naukkarinen et al.[93] and Heinonen et al.[22]. The raw gene expression data were preprocessed with the GeneChip robust multiarray averaging algorithm in the BioConductor package[279] for R using the Brainarray custom cdf[280] for annotation of the probes.

For Study I, based on the literature, we investigated some selected Gene Ontology (GO) terms related to inflammation or immune cell activity. The following GO terms were included in the analyses: 'positive regulation of macrophage chemotaxis' (GO: 0010759), 'macrophage activation involved in immune response' (GO: 0002281), 'positive regulation of neutrophil chemotaxis' (GO: 0090023), 'positive regulation of T cell chemotaxis' (GO: 0010820), and 'positive regulation of acute inflammatory response' (GO: 0002675). Also, 'positive regulation of chronic inflammatory response' (GO: 0002678), 'positive regulation of T cell activation' (GO: 0050870), and 'positive regulation of neutrophil activation' (GO: 1902565) were selected for analysis, but discarded after it was found that many of their genes were not expressed at detectable levels in the samples. A mean centroid value representing the activity of a pathway was calculated for the GO pathways by normalizing the expression levels of the genes in the pathway to a mean of zero and standard deviation of 1 (Z-scoring) across all individuals, and then the arithmetic mean of the Z-scores was calculated to represent the relative activation of that pathway.

For Studies I and II total PARP activity in SAT was measured in a subset of individuals using a HT Colorimetric PARP/Apoptosis Assay (catalog 4684-096-K; Trevigen) according to the manufacturer's instructions. The results were normalized using DNA concentrations measured with a Qubit 2.0 Fluorometer (LifeTechnologies). Also, SIRT1 expression from the microarray was validated against quantitative RT-PCR measurements of SIRT1 mRNA, as described in the original article of Study I.

For Study II, we selected two sets of genes, one depicting inflammation (from Qiagen Human Inflammatory Cytokines and Receptors RT^2 Profiler PCR Array) and one depicting oxidative stress (from Qiagen Human Oxidative Stress RT^2 Profiler PCR Array) (Table 5). We calculated the mean centroid values reflecting average relative activity of the pathways as in Study I.

Table 5. *List of genes used to calculate the inflammation and oxidative stress pathway activities for Study II.*

Inflammation-related genes	Oxidative stress-related genes
CCL13	APOE
CCL2	ATOX1
CCL5	CAT
CCL8	CCL5
CX3CL1	CSDE1
CXCL12	CYGB
CXCL2	DHCR24
CXCL9	DUSP14
CCL13	GLRX2
CCR1	GPX1
CX3CR1	GPX3
IL15	GPX4
IL16	GSS
IL1RN	MSRA
IL33	NME5
IL10RA	NUDT1
IL10RB	OXR1
IL1R1	OXSR1
AIMP1	PDLIM1
BMP2	PNKP
CSF1	PRDX2
MIF	PRDX5
NAMPT	PRDX6
SPP1	PRNP
VEGFA	RNF7
TNFRSF11B	SCARA3
	SEPP1
	SGK2
	SOD1
	SOD2
	SRXN1
	STK25

4.3 STATISTICAL METHODS

All our studies have been performed in the context of null hypothesis significance testing. However, due to high statistical power in Study III, the results are discussed and interpreted with the emphasis not so much on statistical significance, but on effect sizes. Statistical analyses were performed in Stata 12[281] and RStudio[282] (and R[283]). Basic statistics of the data were calculated and the distributions of the variables were assessed mainly with Stata 12. Almost all of the statistical analyses were performed in R. Differences between continuous variables in two groups were compared with *t*-tests. The *t*-tests were either paired or for independent samples with equal variances, as appropriate. The equality of variances was not however evaluated. Wilcoxon signed-rank tests were used to compare paired values in variables with a non-normal distribution. Mann-Whitney *U*-tests were used for non-paired data that were non-normally distributed. In the weight loss study (Study II), the values for variables at 0, 5, and 12 months were analyzed with one-way or two-way repeated measures ANOVA in Stata 12 as appropriate. Global ANOVA P-

values were reported and comparisons between time points were compared by Wald tests.

Generally, normality of variables was assessed either visually via kernel density plots with overlaid normal distributions of the same mean and standard deviation and by calculating the skewness and kurtosis of the variables. To enable the use of parametric statistics (partial correlations in Study I and linear regressions in Study III) in non-normally distributed variables, logarithmic transformations were performed, and the approximate normality of the resulting transformed variables was assessed via kernel density plots. The following variables were $\log_e$-transformed prior to plotting, correlations, or regressions: Study I: sc fat volume, VAT amount, liver fat %, plasma leptin, plasma adiponectin, serum hs-CRP, HOMA-IR, Matsuda index, *SIRT1* expression, *SIRT5* expression, *NAMPT* expression, *CD14* expression, and macrophage activation mean centroid. Study II: VAT amount, liver fat %, plasma triglycerides, HOMA-IR, and Matsuda index. Study III: HOMA-IR, BIGTT-AIR, HDL, triglycerides, fasting glucose, and VAT amount. Additionally in Study III due to extreme skewness, liver fat % was transformed as -1/sqrt(liver fat %), as the next transformation for removing positive skew in the ladder of tranformations[284]. Although variable transformations should perhaps ideally be made in the context of the applied statistical models (e.g. with a Box-Cox procedure for multivariate linear regression), to facilitate interpretation of the analyses and for simplicity, the transformations in the studies of this thesis were made with the goal of achieving univariate normality.

Partial correlations were used to control for age and sex in Studies I and II. In Study I, in the individual-level partial correlation analyses, variance estimates were corrected for stratified sampling by MZ twin pairs by using a mixed model (survey package[285] for R, version 3.29-5). In the individual analyses for Study III, with DZ and MZ twins, the linear regression models were performed with a classical twin regression model for quantitative traits[30] with an ACE decomposition (twinlm function of mets package[286] for R, version 1.1.1). These models take the clustered sampling by twin pairs and their different degrees of genetic similarity into account. The error components model used for the ACE regression used[30] is the following:

$$y_{ij} = \beta_0 + x_{ij1}\beta_1 + \ldots + x_{ijn}\beta_n + A_{ij} + C_{ij} + \varepsilon_{ij} \ ,$$

where i denotes the individual i of pair j, y is the predicted variable, x_1 is the first predictor variable, x_n is the n'th predictor, β_0 is the intercept of the model, β_1 is the regression coefficient for the first predictor, β_n is the regression coefficient for the n'th predictor. The three "error" terms are as follows: $A_{ij} \sim N(0, \sigma_A^2)$ denotes the additive genetic component, $C_{ij} \sim N(0, \sigma_C^2)$ denotes the shared environmental component, and $\epsilon_{ij} \sim N(0, \sigma_E^2)$ denotes the unique environmental component (and measurement error). The covariances for A, C, and E are 1, 1, and 0 for MZ pairs and 0.5, 1, and 0 for DZ pairs, respectively. This model essentially gives similar estimates for the βs as regular linear

regression would had it been applied to the twins as individuals, but as the covariances of the error terms A, C, and E are known for MZ and DZ twins, this mixed model can be used to more precisely model the error terms and take the stratified sampling by MZ and DZ pairs into account.

The MZ twin intrapair differences were calculated differently in Studies I and III. In Study I, 26 of 40 pairs in the sample were sampled to be discordant for BMI (ΔBMI > 3 kg/m^2) from the high end of the BMI distribution, such that one twin was obese or overweight. To minimize the inflation of the estimates of associations between intrapair differences of BMI-associated variables, the heavier twin was always assigned as twin2 and the leaner one as twin1. Thus, the intrapair difference was calculated as Δvariable = variable$_{twin2}$ - variable$_{twin1}$, so that the ΔBMI is always positive.

For Study III, the twin ordering to calculate the intrapair differences was randomized because most the twin pairs were sampled randomly with respect to the intrapair BMI difference. However, because of the randomness associated with twin ordering, a bootstrapping procedure[287] (boot package[288] for R, version 1.3-18) was implemented to estimate the most representative effect sizes, standard errors, P-values and R^2s for the multiple linear regression models. The regression models were bootstrapped by resampling (with replacement) from the original data, after which the sign of the Δ-variables was randomized within pairs. Altogether 10 000 bootstrap samples were analyzed for each regression model, and mean βs and R^2s of the bootstrap samples were reported. Additionally, the 95% confidence intervals of the βs were estimated with the percentile method[287]. Furthermore, we estimated the P-values associated with the β coefficients by running a null hypothesis bootstrap (where we resampled as before, but randomized the observations of each variable across pairs). We then calculated the proportion of effect sizes from the null hypothesis bootstrap that were larger in magnitude than the absolute values of the reported effect size, which we reported as the P-value accordingly. In addition, we combined the βs, confidence intervals, and P-values with a random effects generic inverse variance meta-analysis (metagen package[289] for R, version 1.0), and reported them. This was done to simplify the reporting of results. Although there was statistically significant heterogeneity for some of the estimates (see supplements for the original article of Study III), the results were aggregated for reporting since we found no good reason to assume the estimates from one sample would be more accurate. Individual estimates for each sample can be seen in the supplements for the original article of Study III.

No multiple correction procedures were implemented in any of the studies, thus, the amount of false-positive significant findings is in reality above the 0.05% implicated by the nominal P-values. Generally, Studies I (40 subjects) and II (19 subjects) did not have adequate statistical power to detect small effect sizes, so inferences about them cannot be made. For Study III, with a high number of subjects (total N = 1224), statistical power was not a problem even for small effect sizes. However, in the intrapair differences subanalyses

in Study III with VAT and liver fat % (n = 41 pairs), statistical power was limited; to achieve a power of 0.80, the true effect sizes would have to have been approximately $|r| \geq 0.50$, thus limiting the ability to detect weak and moderate associations. Additionally, in Study III the effect of the bootstrapping procedure on statistical power was not assessed.

5 RESULTS

5.1 ADIPOSE TISSUE SIRTUIN/NAD$^+$ -BIOLOGY AND METABOLIC HEALTH IN ACQUIRED OBESITY (STUDY I)

In Study I, the heavier co-twins of the BMI-discordant pairs were 18.0 kg (25%) heavier and had 13.3 kg (69%) more total adipose tissue, 8.9% higher fat %, and 20% greater waist circumference, compared with their leaner co-twins (Table 6). Greater adiposity in the heavier co-twins was associated with accumulation of metabolically detrimental ectopic fat: they had 172% more visceral fat and 476% more liver fat (although the distributions of these variables have strong positive skewness, producing large relative differences in the untransformed variable). The heavier co-twins reported less physical activity, measured with the Baecke questionnaire (Table 6). Despite differences in adiposity, there were no significant differences in energy, protein, fat, or carbohydrate intake, measured by 3-day food records (Table 6). The heavier co-twins had higher insulin resistance (HOMA-IR), lower insulin sensitivity (Matsuda index), lower plasma adiponectin, higher LDL cholesterol, lower HDL-cholesterol, and higher triglycerides, indicating worse metabolic health (Table 6).

Table 6. *Clinical characteristics of BMI-concordant and BMI-discordant MZ twins in Study I.*

Variable	BMI-concordant both co-twins (*n* = 28)	BMI-discordant lean (*n* = 26)	BMI-discordant heavy (*n* = 26)	P for lean vs. heavy
Age (years)	31.9 ± 3.3	30.2 ± 4.8	30.2 ± 4.8	
% female	36 %	65%	65%	
Height (cm)	171 ± 11	172 ± 10	172 ± 10	0.349
Weight (kg)	79.3 ± 13.3	75.4 ± 18.0	93.3 ± 20.4	<0.001
BMI (kg/m^2)	27.0 ± 3.6	25.3 ± 4.5	31.3 ± 5.2	<0.001
Waist circumference (cm)	87.4 ± 9.3	83.6 ± 12.9	99.8 ± 14.6	<0.001
Body fat (%)	29.2 ± 9.1	32.3 ± 9.2	41.1 ± 6.8	<0.001
Subcutaneous fat (cm^3)	2850 (2420-4000)	3230 (2440-4910)	5710 (4310-7950)	<0.001
Visceral adipose tissue (cm^3)	1040 (448-1480)	571 (327-805)	1200 (743-2210)	<0.001
Liver fat (%)	0.97 (0.51-2.01)	0.58 (0.42-0.97)	2.73 (0.64-7.28)	<0.001
Fat free mass (kg)	55.9 (45.8-61.2)	46.0 (41.2-57.7)	51.7 (41.9-63.9)	<0.001
Adipocyte diameter (µm)[a]	84.0 ± 11.0	80.9 ± 12.7	95.0 ± 14.1	<0.001
fP-glucose (mmol/L)	5.40 (4.90-5.60)	5.05 (4.90-5.50)	5.40 (4.90-5.70)	0.059
AUC glucose (mmol/l × h)	13.9 (12.1-15.9)	13.8 (12.5-15.3)	14.6 (13.3-17.3)	0.728
fS-insulin (mU/L)	5.2 (3.0-6.9)	4.5 (3.2-6.7)	7.5 (4.9-9.9)	<0.001
AUC insulin (pmol/l × h)	66.2 (47.7-86.0)	76.5 (58.1-103)	111 (86.3-134)	0.047
HOMA-IR	1.33 (0.64-1.72)	1.05 (0.69-1.51)	1.75 (1.02-2.38)	<0.001
Matsuda index	7.51 (5.86-12.4)	8.01 (6.04-11.2)	4.82 (3.92-7.57)	0.001
fP-Leptin (ng/mL)	7.5 (3.7-15.2)	12.2 (7.5-27.1)	27.0 (18.8-55.7)	<0.001
fP-Adiponectin (µg/mL)	2.5 (1.9-4.0)	3.7 (2.4-4.8)	2.6 (1.8-3.5)	<0.001
fS-eNampt (ng/ml)	3.06 ± 1.71	2.87 ± 1.48	3.24 ± 1.67	0.357
fS-hs-CRP (mg/dL)	0.80 (0.32-1.48)	1.01 (0.45-3.93)	1.58 (0.8-6.32)	0.015
LDL-cholesterol (mmol/L)	2.80 (2.35-3.35)	2.55 (2.10-2.90)	2.75 (2.50-3.40)	0.035
HDL-cholesterol (mmol/L)	1.24 (1.09-1.50)	1.64 (1.28-1.94)	1.27 (1.12-1.42)	<0.001
Triglycerides (mmol/L)	0.72 (0.57-1.11)	0.87 (0.68-1.17)	1.13 (0.78-1.43)	0.014
Physical activity	8.65 ± 1.89	8.64 ± 1.51	7.99 ± 1.42	0.037
Energy intake (kcal)	2180 ± 550	2070 ± 543	2130 ± 531	0.663
Protein intake (g)	97.9 ± 33.4	90.0 ± 38.4	86.2 ± 25.4	0.674
Fat intake (g)	82.6 ± 27.6	82.8 ± 30.8	84.8 ± 22.5	0.764
Carbohydrate intake (g)	229 ± 62.9	220 ± 61.3	222 ± 70.3	0.899

Data are presented as "mean ± SD", and "median (interquartile range)" for skewed variables. Comparisons were made with paired *t*-tests and Wilcoxon signed-rank tests as appropriate. [a)]For adipocyte diameter *n* = 16 for concordant and *n* = 25 for discordant pairs. Adapted from Jukarainen et al.[276].

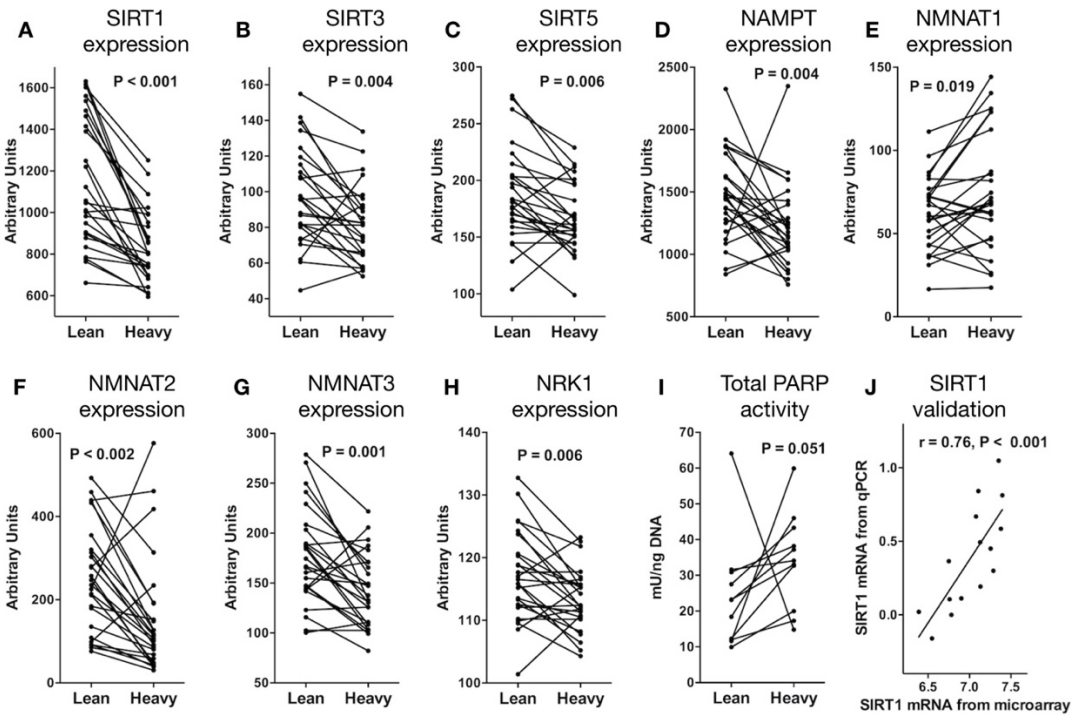

Figure 11 Expression of SIRTs and NAD$^+$ biosynthesis genes in BMI-discordant MZ twin pairs. The expression of SAT *SIRT1* (A), *SIRT3* (B), *SIRT5* (C), *NAMPT* (D), *NMNAT1* (E), *NMNAT2* (F), *NMNAT3* (G), and *NRK1* (H) expression in lean and heavy co-twins of MZ twin pairs discordant for obesity (ΔBMI > 3 kg/m^2, n=26). SAT total PARP activity in a subset of BMI-discordant MZ twin pairs (I, *n* = 22). *SIRT1* expression from microarray validated against SIRT1 expression from RT-PCR in a subset of twins (J, *n* = 15 individuals). Significance testing with Wilcoxon signed-rank test (A-I) and Pearson's correlation (J). SIRT1-5, sirtuin 1-5; NAMPT, nicotinamide phosphoribosyltransferase; NMNAT1–3, nicotinamidemononucleotide adenylyltransferase 1–3; NRK1, nicotinamide riboside kinase 1; PARP, poly ADP ribose polymerase. Adapted from Jukarainen et al.[276].

Comparing the expressions of sirtuin and NAD$^+$ biosynthesis genes from the Affymetrix microarray in BMI-discordant MZ twins with Wilcoxon signed-rank tests revealed that *SIRT1* (P < 0.001), *SIRT3* (P = 0.004), and *SIRT5* (P = 0.006) were expressed at significantly lower levels in the heavier co-twins (Figure 11A-C). No significant differences were found in the expressions of *SIRT2* or *SIRT7* (data not shown), whereas the expressions of *SIRT4* and *SIRT6* were undetectable. Next, we examined the expressions of genes involved in NAD$^+$ biosynthesis since the enzymatic activity of sirtuins depends on cellular NAD$^+$ levels. *NAMPT* (P = 0.004), *NMNAT2* (P = 0.002), *NMNAT3* (P = 0.001), and *NRK1* (P = 0.006) expressions were significantly lower in heavier co-twins, whereas *NMNAT1* (P = 0.019) expression was higher (Figure 11D-H). Additionally, the total activity of PARP proteins, the most important consumers of intracellular NAD$^+$, was measured for 9 pairs. There was a trend for higher total PARP activity in the heavier co-twins (Figure 11I). We also

measured the concentrations of serum NAMPT (visfatin), but no significant differences were observed within pairs, despite a moderate correlation between serum NAMPT and BMI (r = 0.25, P = 0.028) or VAT amount (r = 0.31, P = 0.006) in the individuals (data not shown). In addition, we verified the expression of *SIRT1* with RT-PCR in a subset of twins. The expression of SIRT1 determined by the Affymetrix microarray and RT-PCR were highly correlated (r = 0.76, P < 0.001, Figure 11J). The localizations of SIRT1, SIRT3, and SIRT5 to adipocytes was verified with immunohistochemical stains (Study I, Supplemental Figure 1). The relationship between SIRT1 and BMI seems to have a floor effect, with values of SIRT1 plateauing at higher BMIs, however, after $\log_e$-transformation, the relationship seems to be closer to linear (Figure 12). However, the local polynomial regression (LOESS) fit is not reliable at the ends of the BMI distribution due to low numbers of observations, but it is suggestive of a nonlinear association relative to the linear fit (Figure 12).

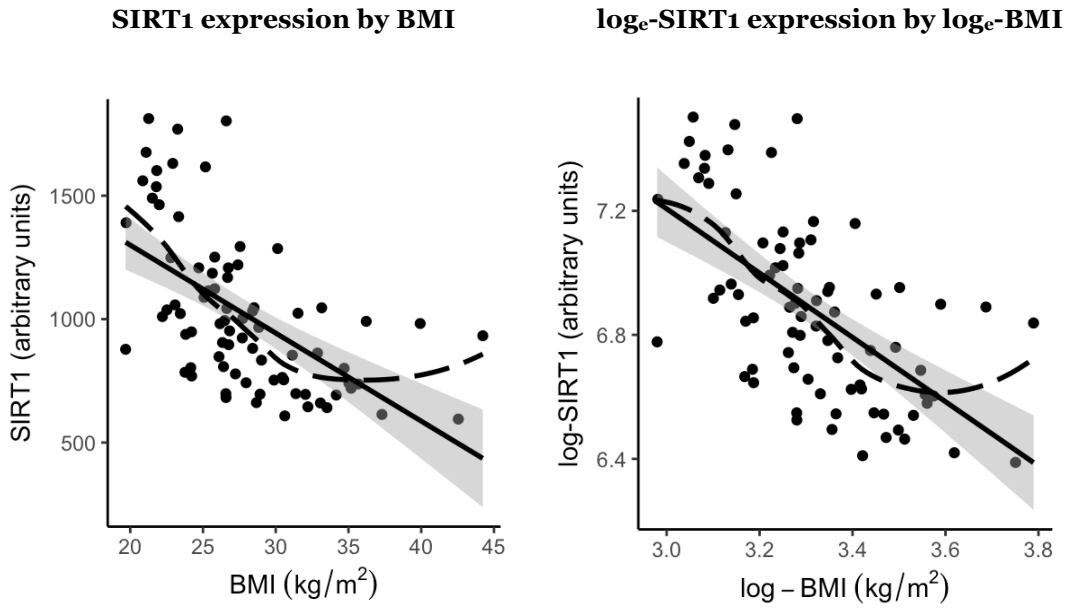

Figure 12 SIRT1 expression by BMI untransformed and $\log_e$-transformed plots. The solid black line represents a linear least squares fit with the shaded area as 95 % confidence intervals of the fit, the dashed line represents a local polynomial regression fit (LOESS). Unpublished results.

To further examine how the downregulation of SIRT and NAD^+ biosynthesis gene expression in the heavier co-twins' SAT correlates with different measures of adiposity, inflammation, and insulin resistance, we calculated partial correlations at the individual level and for intrapair differences in variables, using all 40 available twin pairs, including the BMI-concordant twins. The correlations were adjusted for age and sex. When studying MZ twins, the correlations between intrapair differences in variables reflect the unique environmental correlation between those variables, and thus, are not confounded by genetic or shared environmental factors. When looking at

twins as individuals, *SIRT1* expression was negatively correlated with multiple variables depicting adiposity: BMI, body fat %, SAT volume, IA fat volume, liver fat %, and adipocyte diameter (Figure 13). Intrapair differences in these variables had similar correlations with *SIRT1*. *SIRT3* was correlated negatively with BMI, body fat %, SAT volume, and IA fat volume in individuals and showed intrapair differences. Additionally, *SIRT5* was correlated negatively with all of the adiposity variables in individuals, but not intrapairs. NAD^+ biosynthesis genes were similarly negatively correlated with the mentioned adiposity variables in individuals (Figure 14), except for *NMNAT1* (results not shown). In examination of intrapair differences, *NAMPT*, *NMNAT3,* and *NRK1* were correlated with most of the adiposity variables (Figure 14).

Sirtuins are linked to the regulation of glucose metabolism and insulin sensitivity. We thus went on to examine the correlations of sirtuin protein expression with insulin sensitivity indices derived from an oral glucose tolerance test. In individuals, *SIRT1* had significant positive correlations with the Matsuda index and negative correlations with HOMA-IR (Figure 13). Intrapair analysis revealed *SIRT1* to be significantly negatively correlated with HOMA-IR intrapair. In individuals, *SIRT3* expression had a positive correlation with HOMA-IR and a negative correlation with Matsuda index, but these associations were not significant intrapair. *SIRT5* expression was associated negatively with HOMA-IR and positively with Matsuda index in individuals, but only the association with HOMA-IR was significant intrapair. None of the *SIRTs* were consistently associated with plasma adiponectin levels or SAT *ADIPOQ* expression either in individuals or intrapair, despite some significant positive associations (Figure 13). In individuals, all NAD^+ biosynthesis genes, apart from *NMNAT1* (results not shown), had significant positive correlations with the Matsuda index and negative correlations with HOMA-IR (Figure 14). These associations were not, however, significant when analyzing intrapair differences. *NMNAT2*, *NMNAT3*, and *NRK1* expressions were significantly positively associated with plasma adiponectin levels and SAT *ADIPOQ* expression (Figure 14).

SIRT1 and *SIRT3* have been shown to be involved in regulating inflammation and macrophage recruitment to SAT, and thus, we examined serum hs-CRP, *CD14* (a macrophage marker) expression and five gene ontology pathways associated with macrophage chemotaxis (GO: 0010759), macrophage activation (GO: 0002281), acute inflammatory response (GO: 0002675), and T-cell chemotaxis (GO: 0010820) in SAT. *SIRT1*, *SIRT3,* and *SIRT5* were significantly negatively correlated with all of the mentioned inflammatory markers in individuals, and with *SIRT1* and *SIRT5* in pairs (Figure 13). All of the NAD^+ biosynthesis genes, apart from *NMNAT1* (results not shown here), had negative correlations with the majority of the inflammatory markers in individuals (Figure 14). However, only *NMNAT2*, *NMNAT3,* and *NRK1* had significant negative intrapair correlations (Figure 14).

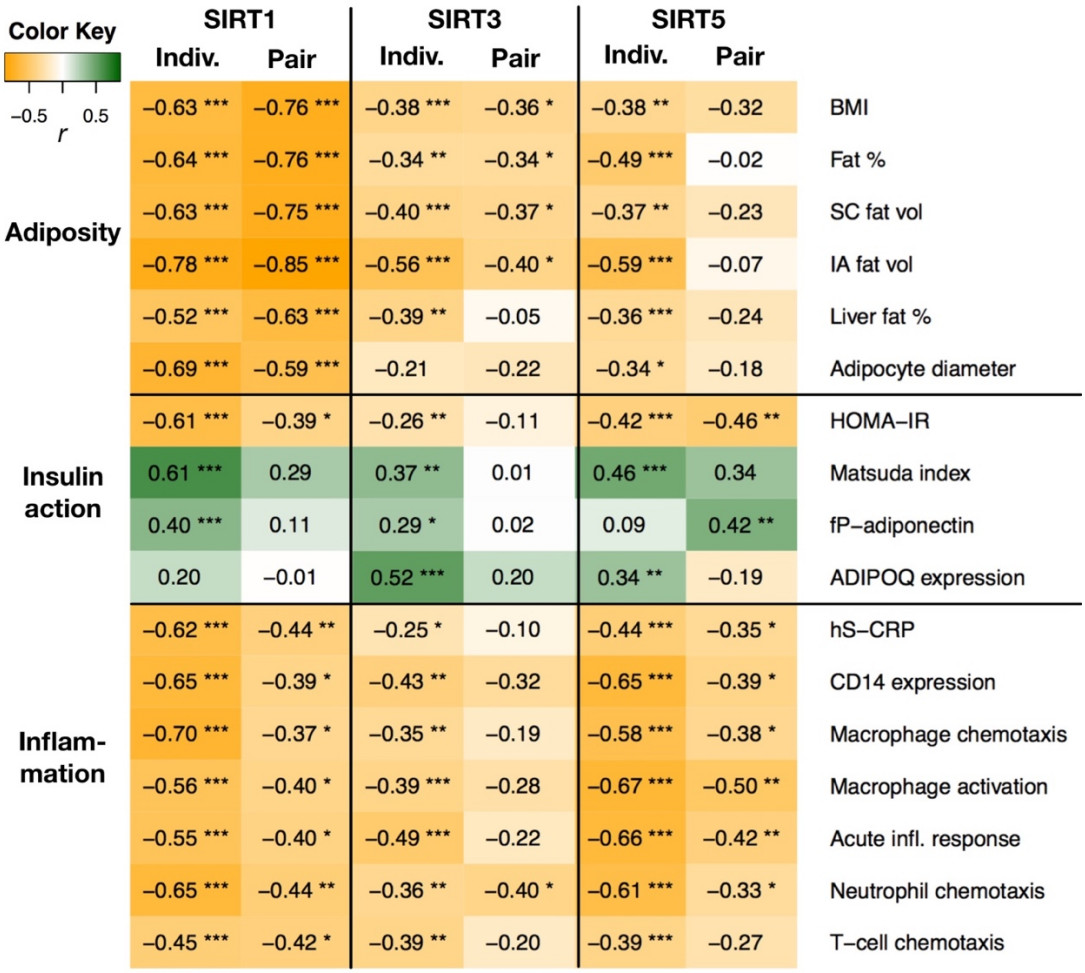

Figure 13 Correlations of SAT *SIRT1*, *SIRT3*, and SIRT5 expression with different clinical or SAT expression variables related to adiposity, insulin action, and inflammation in all MZ twins. Coefficients depict partial correlations between variables after adjusting for age and sex. Individual (Indiv.) partial correlations are calculated by treating every subject as an individual (*N* = 80), and the associated P-values are corrected for stratified sampling by twin pairs using a mixed model. Within-pair (Pair) correlations are partial correlations calculated for within-pair differences (heavy - lean co-twin) of each variable (*n* = 40). The colors of the cells denote the magnitude and direction of the correlations (orange = negative, green = positive). Asterisks indicate statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001. For the last 5 rows, see Methods section 4.2.5 for how the mean centroid values for the pathways were calculated. ADIPOQ, adiponectin gene; fP-adiponectin, fasting plasma adiponectin level; hS-CRP, high sensitivity C-reactive protein assay; SC fat vol, subcutaneous fat volume; IA fat vol, intra-abdominal fat (visceral adipose tissue) volume. Adapted from Jukarainen et al.[276].

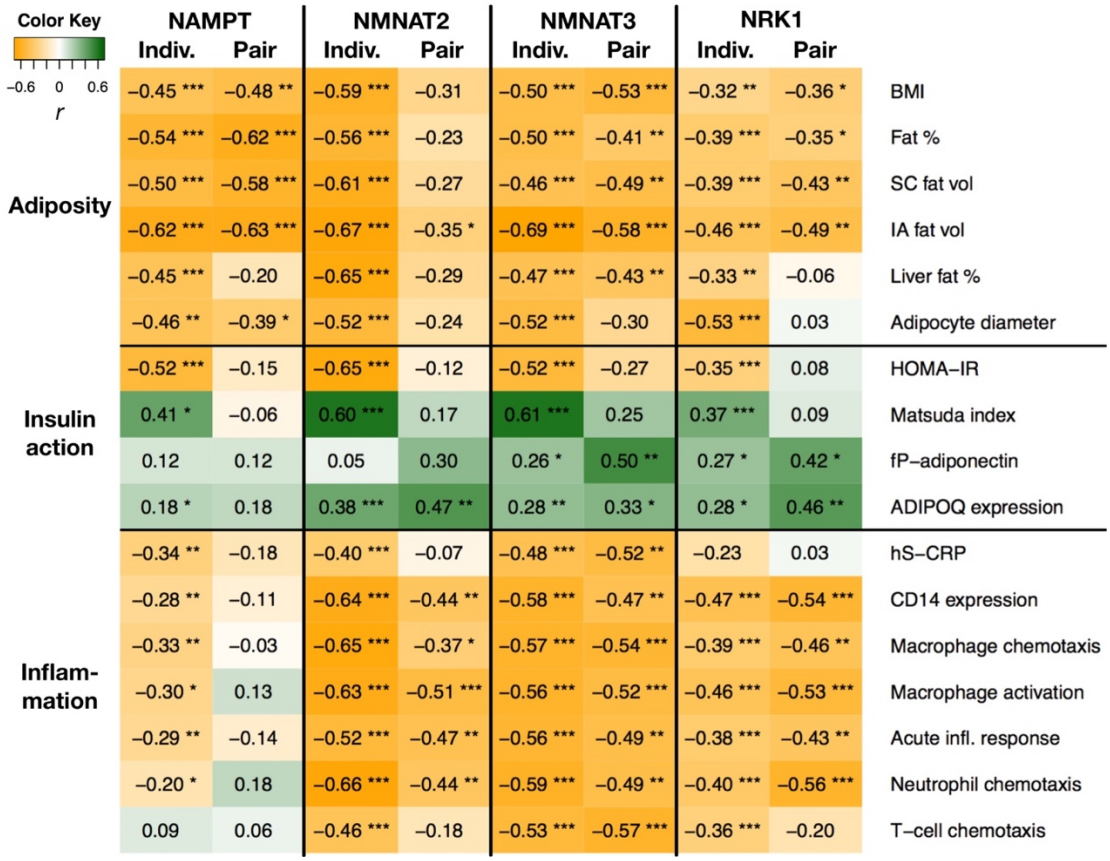

Figure 14 Correlations of SAT *NAMPT*, *NMNAT2*, *NMNAT3*, and *NRK1* expression with different clinical or SAT expression variables related to adiposity, insulin action, and inflammation in all MZ twins. Coefficients depict partial correlations between variables after adjusting for age and sex. Individual (Indiv.) partial correlations are calculated by treating every subject as an individual (N = 80), and the associated P-values are corrected for stratified sampling by twin pairs using a mixed model. Within-pair (Pair) correlations are partial correlations calculated for within-pair differences (heavy - lean co-twin) of each variable (n = 40). The colors of the cells denote the magnitude and direction of the correlations (orange = negative, green = positive). Asterisks indicate statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001. For the last 5 rows, see Methods section 4.2.5 for how the mean centroid values for the pathways were calculated. ADIPOQ, adiponectin gene; fP-adiponectin, fasting plasma adiponectin level; hS-CRP, high sensitivity C-reactive protein assay; NAMPT, nicotinamide phosphoribosyltransferase gene; NMNAT2-3, nicotinamide mononucleotide adenylyltransferase 2-3 genes; NRK1, nicotinamide riboside kinase 1 gene; SC fat vol, subcutaneous fat volume; IA fat vol, intra-abdominal fat (visceral adipose tissue) volume. Adapted from Jukarainen et al.[276].

UPR^{mt}, a mitochondrial proteostasis pathway, has been thought to be regulated by sirtuins (especially *SIRT1*). Thus, we further examined the expressions of nine UPR^{mt}-related genes in the twin pairs discordant for BMI

(Figure 15). Of these genes, the expressions of *DDIT3* (DNA damage inducible transcript 3, also known as *CHOP*), *HSPD1* (heat shock 60kDa protein 1, also known as *HSP60*), *CLPP* (caseinolytic mitochondrial matrix peptidase proteolytic subunit), and *HTRA2* (HtrA serine peptidase 2) were significantly lower in heavier co-twins (Figure 15).

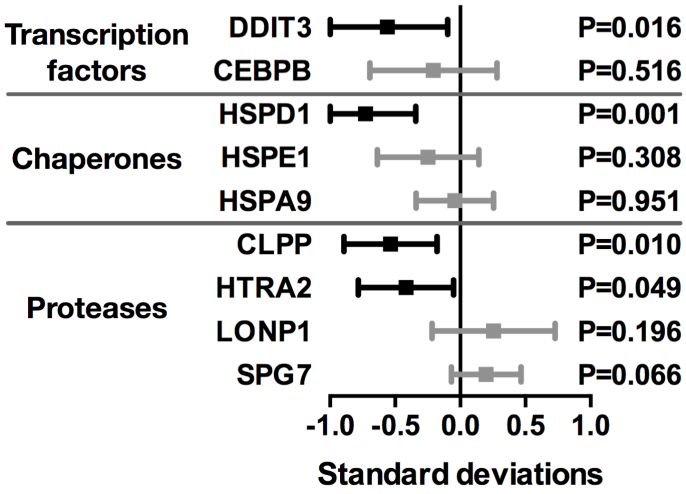

Figure 15 Comparison of SAT expression values of genes related to the UPR^{mt} pathway in lean and heavy co-twins of BMI-discordant MZ twin pairs (*n* = 26). The squares (mean within-pair differences) and bars (95% confidence intervals) depict the Z-scored leaner co-twin's value minus the Z-scored heavier co-twin's value. P-values are from pairwise *t*-tests. *CEBPB*, CCAAT/enhancer-binding protein beta; *CLPP*, caseinolytic mitochondrial matrix peptidase proteolytic subunit; *DDIT3*, DNA damage inducible transcript 3; *HSPA9*, heat shock 70kDa protein 9; *HSPD1*, heat shock 60 kDa protein 1; *HSPE1*, heat shock 10kDa protein 1; *HTRA2*, HtrA serine peptidase 2; *LONP1*, Lon peptidase 1, mitochondrial; *SPG7*, spastic paraplegia 7. Adapted from Jukarainen et al.[276].

5.2 ADIPOSE TISSUE SIRTUIN/NAD$^+$ -BIOLOGY AND METABOLIC HEALTH IN WEIGHT LOSS (STUDY II)

In Study II, obese weight loss program subjects were at baseline on average 47% heavier (99.0 kg vs. 67.4 kg), had 52% higher BMI (34.6 kg/m^2 vs. 22.7 kg/m^2), 48% greater waist circumference (112.5 cm vs. 76.2 cm), and 19.3% higher fat % (Table 7) than the unrelated lean reference subjects. Greater adiposity in the obese weight loss program subjects was associated with higher fasting glucose, insulin resistance (HOMA-IR), lower insulin sensitivity (Matsuda index), lower HDL, higher triglycerides, higher systolic and diastolic blood pressure, and higher liver fat % compared with reference subjects.

Table 7. *Clinical and metabolic characteristics of lean reference subjects at baseline and of obese subjects at baseline and at the follow-ups.*

Variables	Reference subjects (*n* = 19)	Obese subjects (*n* = 19)			P-values[a]			
	0 Mo	0 Mo	5 Mo	12 Mo	obese vs. refe-rence	obese 0-5 Mo	obese 5-12 Mo	obese 0-12 Mo
Weight (kg)	67.4 (2.6)	99.0 (3.2)	87.4 (3.3)	90.1 (3.4)	0.001	<0.001	0.015	<0.001
Height (cm)	171.5 (2.8)	168.7 (2.2)			0.4431			
BMI (kg/m^2)	22.7 (0.3)	34.6 (0.6)	30.6 (0.8)	31.6 (3.9)	<0.001	<0.001	0.011	<0.001
Body fat (%)	25.3 (1.9)	44.6 (1.6)	39.6 (2.0)	40.6 (9.3)	<0.001	<0.001	0.170	<0.001
Waist (cm)	76.2 (1.5)	112.5 (2.3)	100.1 (2.6)	99.4 (11.8)	<0.001	<0.001	0.422	<0.001
Adipocyte diameter (µm)	75.4 (2.4)	102.1 (1.9)	97.8 (2.4)	100.4 (3.5)	<0.001	0.150	0.133	0.648
fP-glucose (mmol/L)	5.1 (0.1)	5.6 (0.10)	5.4 (0.1)	5.4 (0.7)	<0.001	0.013	0.676	0.046
HOMA index	0.7 (0.4-1.3)	1.9 (1.5-3.3)	1.9 (1.0-2.5)	1.6 (1.3-3.2)	<0.001	0.135	0.509	0.244
Matsuda index	9.7 (6.6-17.2)	4.5 (2.7-6.2)	6.6 (4.6-11.0)	5.9 (4.7-8.0)	<0.001	0.006	0.418	0.235
LDL (mmol/L)	2.8 (0.2)	2.9 (0.1)	2.5 (0.1)	2.6 (0.6)	0.573	0.006	0.562	0.013
HDL (mmol/L)	1.7 (0.1)	1.4 (0.1)	1.4 (0.1)	1.5 (0.3)	0.019	0.379	0.104	0.027
Triglycerides (mmol/L)	0.7 (0.6-1.0)	0.9 (1.5-3.3)	0.7 (0.5-0.9)	0.8 (0.6-1.1)	0.006	0.001	0.070	0.014
Systolic BP (mmHg)	119.8 (1.9)	135.1 (3.4)	117.8 (2.2)	125.1 (2.9)	<0.001	<0.001	0.006	0.002
Diastolic BP (mmHg)	74.7 (1.6)	83.8 (1.9)	81.3 (1.8)	82.1 (2.7)	<0.001	0.269	0.692	0.407
Energy intake (kcal/day)	2240 (109)	2460 (212)	1590 (110)	2010 (183)	0.353	<0.001	0.040	0.090
SAT (kg)		13.8 (0.8)	9.8 (0.9)			<0.001		
VAT (kg)		3.7 (0.6)	2.3 (0.4)			<0.001		
Liver fat (%)	0.67 (0.08)	6.7 (1.1)	1.8 (0.5)		<0.001	<0.001		

Data is presented as "mean (SE)" and for skewed variables "median (interquartile range)". [a]Comparisons were made with unpaired *t*-tests and Mann-Whitney *U*-tests for differences between reference and obese subjects at baseline as appropriate, whereas comparisons between different time points in the obese subjects were made with paired *t*-tests and with Wilcoxon signed-rank tests as appropriate. VAT, Visceral adipose tissue; SAT, subcutaneous adipose tissue. Adapted from Rappou et al.[270].

Between 0 and 5 months, the total energy intake of weight loss study subjects decreased on average by 35.2%, which resulted in a weight loss of 11.6 ± 1.3 kg (-11.7%) along with improvements in fasting glucose, HOMA-IR, Matsuda index, LDL cholesterol, triglyceride levels, and systolic blood pressure (Table 7). Between 5 and 12 months, mean total energy intake increased by 26.4%, with an associated mean weight regain of 2.7 kg. Total weight loss from 0 months to the end of the intervention at 12 months was 9.1 kg (-9.0%). We also assessed the size and metabolic activity of different fat depots at 0 and 5 months. Amounts of VAT, SAT, and liver fat % showed a statistically significant decline from 0 to 5 months (Table 7).

Only 6 of the 19 patients continued to lose weight from 5 months to 12 months, with a total mean weight loss of 17.5 kg (-17.1%) from 0 to 12 months, whereas the other 13 patients, who regained some weight between 5 and 12 months, had a mean total weight loss of 4.9 kg (-5.1%) at 12 months (Figure 16). In continuous weight losers and weight regainers, we observed significant decreases in weight % from baseline and BMI (Figure 16). Adipocyte diameter was decreased at 5 and 12 months in the continuous weight loss group, but there were no significant changes in the weight regainers (Figure 16).

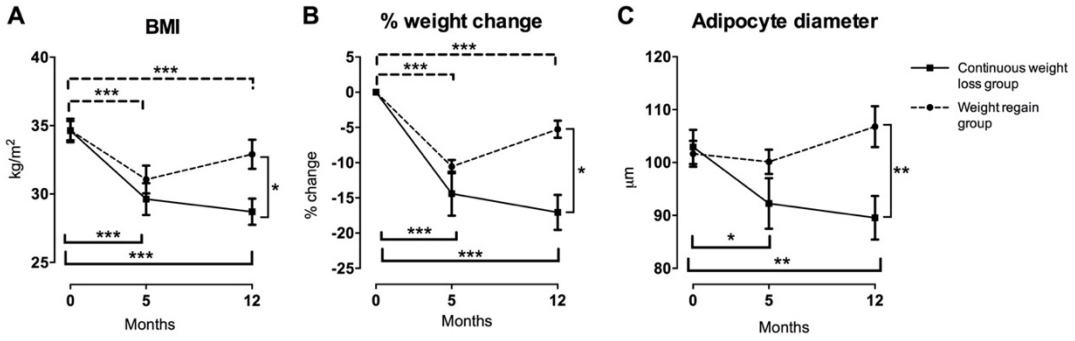

Figure 16 BMI, % weight loss, and mean adipocyte diameter changes of obese subjects before and during the long-term weight loss program in two separate groups: continuous weight losers (n=6, solid line) and weight regainers (n=13, dashed line). BMI (A), % weight loss (B), and adipocyte diameter (C). Data are presented as group mean ± SE. *P<0.05, **P<0.01 ***P<0.001, P-values were calculated with Wald tests as post hoc tests for two-way repeated measures ANOVA. Adapted from Rappou et al.[270].

Next, we examined the SAT gene expression patterns of sirtuins and NAMPT, and SAT total PARP activity. *SIRT1* (P < 0.001), *SIRT3* (P < 0.001), *SIRT7* (P < 0.001), and *NAMPT* (P < 0.05) expression was significantly higher in the lean reference subjects than in the obese weight loss study participants at baseline (Figure 17A-F). There were no significant differences in *SIRT2* and *SIRT5* expression. Additionally, total PARP activity was significantly higher in the obese subjects than in the lean controls (P < 0.05) (Figure 17G). The

microarray did not provide an analyzable expression signal for *SIRT4* and *SIRT6* (data not shown).

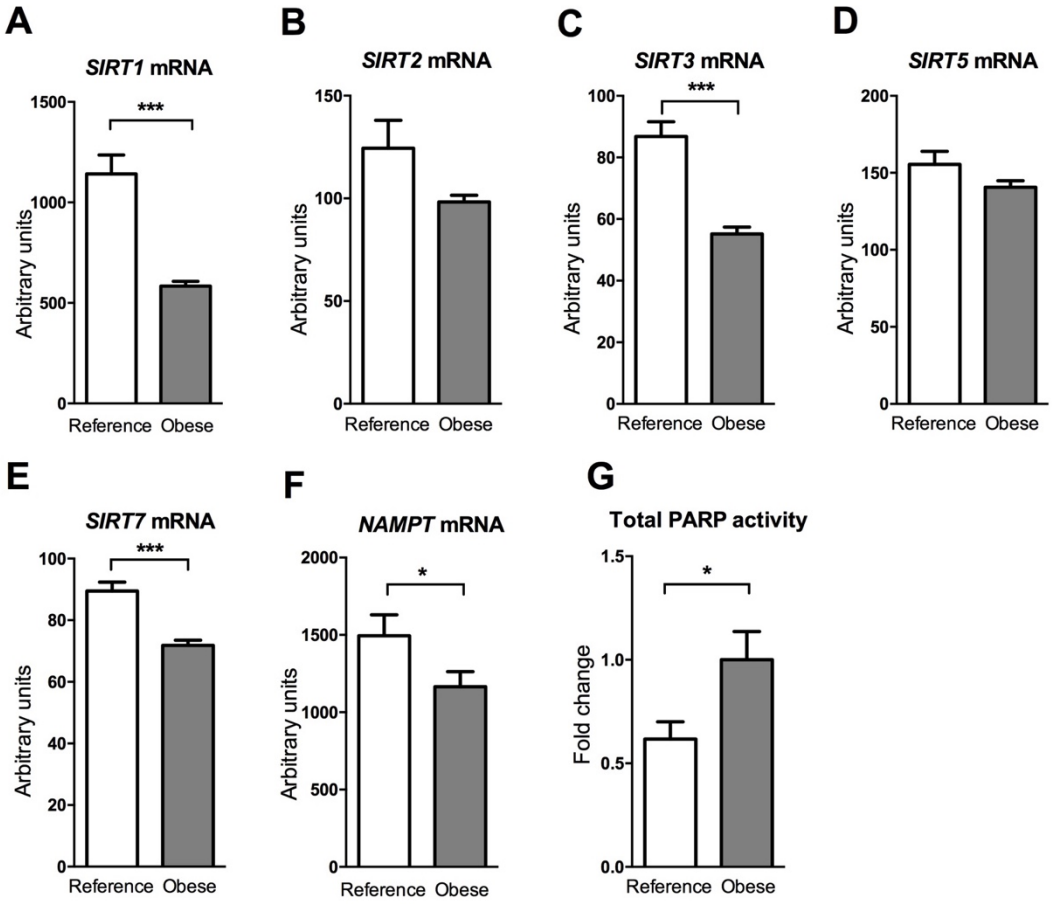

Figure 17 *SIRT* and *NAMPT* mRNA levels and total PARP activity in subcutaneous adipose tissue at baseline (0 months) between two study groups: reference group (white column) and obese subjects (gray column). *SIRT1* (A), *SIRT2* (B), *SIRT3* (C), *SIRT5* (D), *SIRT7* (E), and *NAMPT* (F) mRNA levels, arbitrary units denote microarray mRNA values after normalization. Total PARP enzyme activity (n=11 reference and n=16 obese subjects) (G). Data shown as mean ± SE. *P<0.05, **P<0.01, ***P<0.001. Significance testing was done with unpaired *t*-tests. Adapted from Rappou et al.[270].

Next, we examined how sirtuin gene expressions changed in response to weight loss in all subjects together. *SIRT1* expression was increased in SAT from baseline to 5 and 12 months (Figure 18A). From 0 to 12 months, *SIRT3* expression decreased (Figure 18C) and *SIRT7* expression increased (Figure 18E). There were no significant changes in the expressions of *SIRT2* (Figure 18B) and *SIRT5* (Figure 18D).

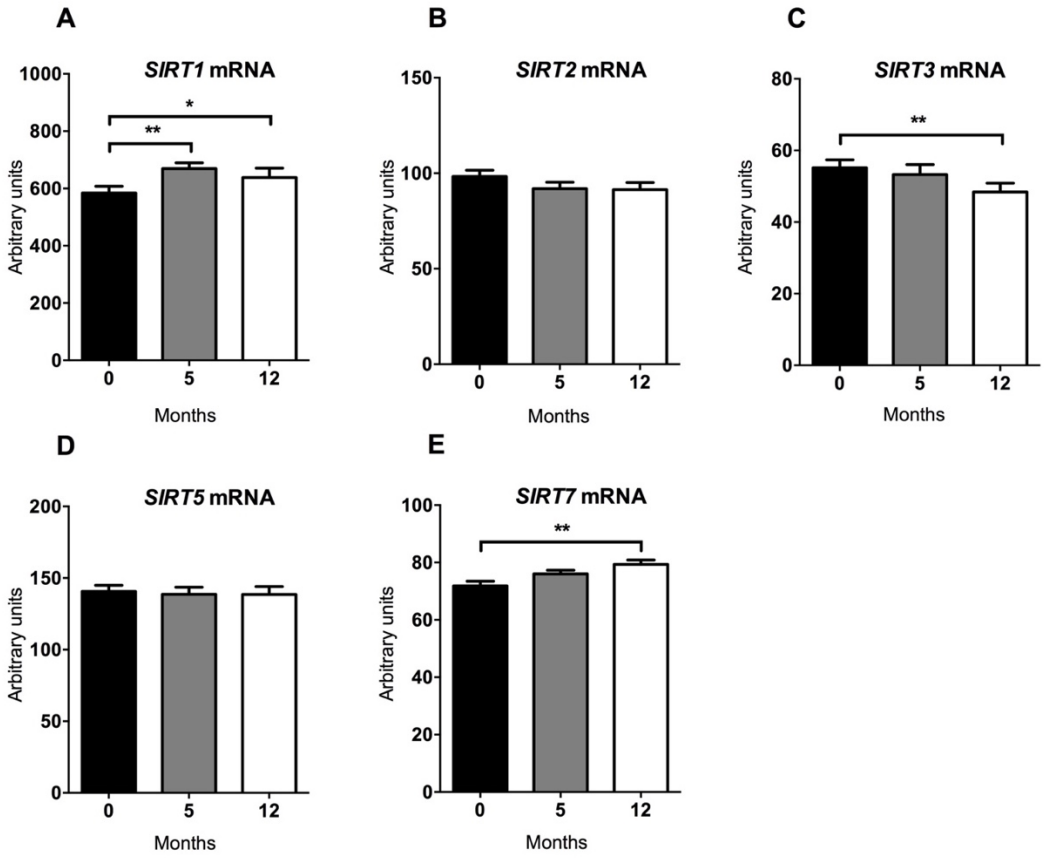

Figure 18 Expression levels of *SIRT*s and *NAMPT,* and total PARP activity in SAT during weight loss in obese subjects. *SIRT1* (A), *SIRT2* (B), *SIRT3* (C), *SIRT5* (D), and *SIRT7* (E) mRNA levels in all study subjects (n=19) during the 12-month weight loss intervention, arbitrary units denote microarray mRNA values after normalization. Data are presented as mean ± SE. *P<0.05, **P<0.01, ***P<0.001. P-values were calculated with Wald tests as post hoc tests for repeated measures ANOVA. Adapted from Rappou et al.[270].

Additionally, we looked at *SIRT1* expression in the two subgroups of continuous weight losers and weight regainers. *SIRT1* expression was significantly higher at baseline among the continuous weight losers (Figure 19A). *SIRT1* expression increased during weight loss from 0 to 5 months in both subgroups, but further increased only in the continuous weight loss group (Figure 19A). There was no significant difference between *SIRT1* expression in weight regainers between 0 and 12 months since the *SIRT1* expression seemed to revert to baseline levels following weight regain (Figure 19A). The expression levels of *SIRT1* seemed to inversely follow the same trend as % weight change or BMI during the follow-up (Figure 16A-B). No clear trends emerged in the two subgroups for other *SIRTs* (data not shown). As sirtuins and PARPs are NAD^+-dependent enzymes, we looked at expression of *NAMPT* and observed that it was significantly higher at 5 months than at baseline, but returned to baseline levels at 12 months (Figure 19B). The expression of *PARP1*

was significantly lower at 12 months than at baseline, whereas the expression of *PARP2* was not significantly changed (Figure 19C-D). We additionally measured total PARP activity of SAT and observed that it was significantly decreased (-38.2%) from baseline to 12 months (Figure 19E).

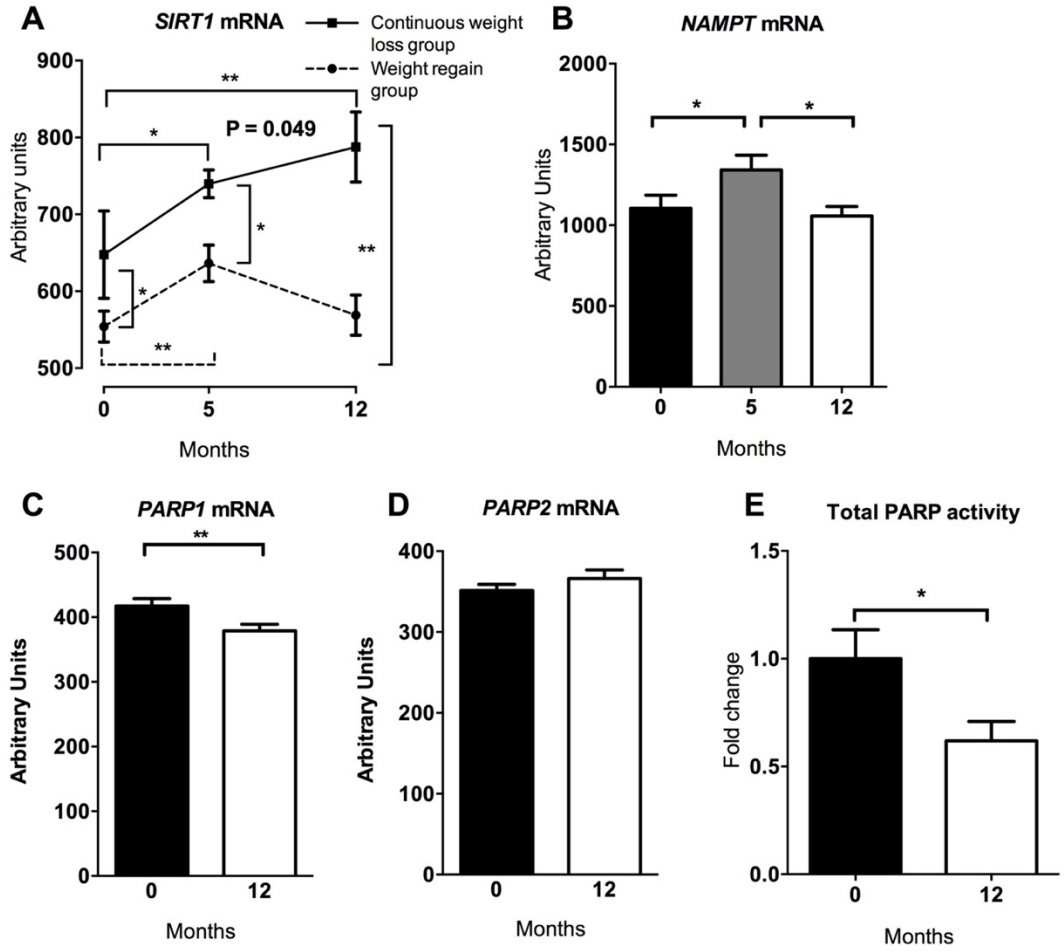

Figure 19 *SIRT1*, *NAMPT*, *PARP1*, and *PARP2* mRNA levels, and total PARP activity in SAT. *SIRT1* (A) mRNA levels are presented in the two separate groups of weight loss subjects (G): continuous weight losers (solid line, *n* = 6) and weight regainers (dashed line, *n* = 13), arbitrary units denote microarray mRNA values after normalization. Total PARP activity (E) was assessed for a subset of the subjects (n=13). Significance testing was performed with Wald tests as post hoc tests for two-way repeated measures ANOVA (A) or one-way repeated measures ANOVA (B), or with paired *t*-tests (C-E). Adapted from Rappou et al.[270].

At baseline, in both lean reference subjects and obese weight loss study participants *SIRT1* expression in SAT was negatively correlated with body fat % and liver fat % (Figure 20A-B). In all subjects, baseline *NAMPT* expression was significantly negatively correlated with fat % (Figure 20C). SAT total PARP activity was significantly positively correlated with fat % in a subset of

individuals from the obese weight loss intervention group that had samples left for this analysis (Figure 20D).

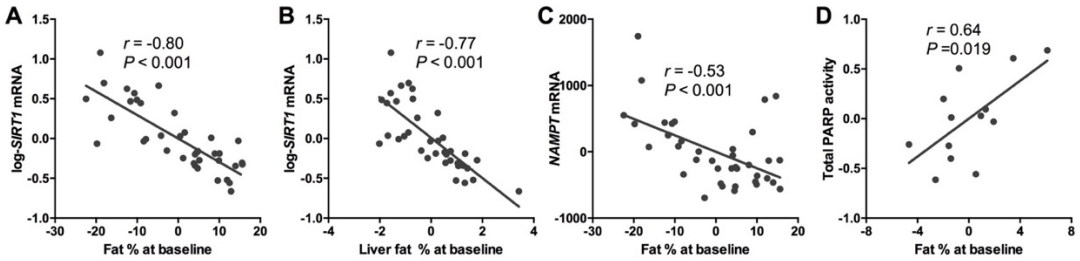

Figure 20 Continuous relationships of baseline fat % and $\log_e$-liver fat % with baseline $\log_e$-*SIRT1* expression, *NAMPT* expression, and total PARP activity. Figures represent partial regression plots controlled for age and sex (residuals of each variable after regressing them on age and sex). Correlation of residual $\log_e$-*SIRT1* mRNA with fat % (A, *n* = 38) and liver fat % (B, *n* = 38) in all subjects at baseline. Correlation of residual *NAMPT* mRNA with fat % (C, *n* = 38) in all subjects at baseline. Correlation between residual total PARP activity and fat % at baseline in a subset of participants from the obese weight loss intervention group that had samples left for this analysis (D, *n* = 13). Effect sizes (*r*) are Pearson's correlations, the solid lines correspond to the linear least squares fit. Adapted from Rappou et al.[270].

In addition to the continuous associations at baseline, we examined the relationship of longitudinal fat % and liver fat % changes with changes in *SIRT1* and *NAMPT* expression (Figure 21A-D). Change in fat % from 0 to 12 months was not significantly associated with change in *SIRT1* (Figure 21A) or *NAMPT* expression (Figure 21C). However, there was a nonsignificant trend (P = 0.066) for a negative correlation between fat % change and SIRT1 expression change (Figure 21A). Change in liver fat % from 0 to 5 months did not correlate with the change in *SIRT1* (Figure 21B) or *NAMPT* expression (Figure 21D).

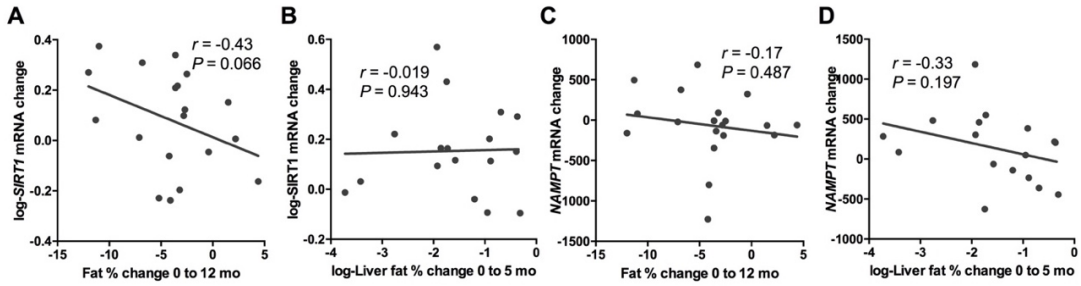

Figure 21 Continuous relationships between longitudinal change in fat % or log_e-liver fat % and log_e-*SIRT1* or *NAMPT* expression. Associations between longitudinal changes in variables for the obese weight loss intervention participants (A-D, *n* = 19). Effect sizes (*r*) are Pearson's correlations with associated P-values, the solid lines corresponds to the linear least squares fit. Adapted from Rappou et al.[270].

We additionally examined inflammation and oxidative stress-related gene expression pathways in SAT. Compared with lean reference subjects, there was higher expression in inflammation-related genes in the obese weight loss study subjects (Figure 22A), but no significant difference emerged for the oxidative stress related genes (Figure 22E). Weight loss did not have a significant effect on the expression of inflammation related genes (Figure 22B), but the expression of oxidative stress-related genes diminished in obese subjects after weight loss at 5 and 12 months (Figure 22F). In addition, the expression of inflammation-related genes in SAT was negatively correlated with SAT *SIRT1* expression (Figure 22D), but not with SAT *PARP1* expression (Figure 22C). *PARP1* expression was positively correlated with the expression of oxidative stress-related genes (Figure 22G). A nonsignificant trend for a negative correlation between the oxidative stress-related genes and *SIRT1* expression was observed (Figure 22H, P = 0.053).

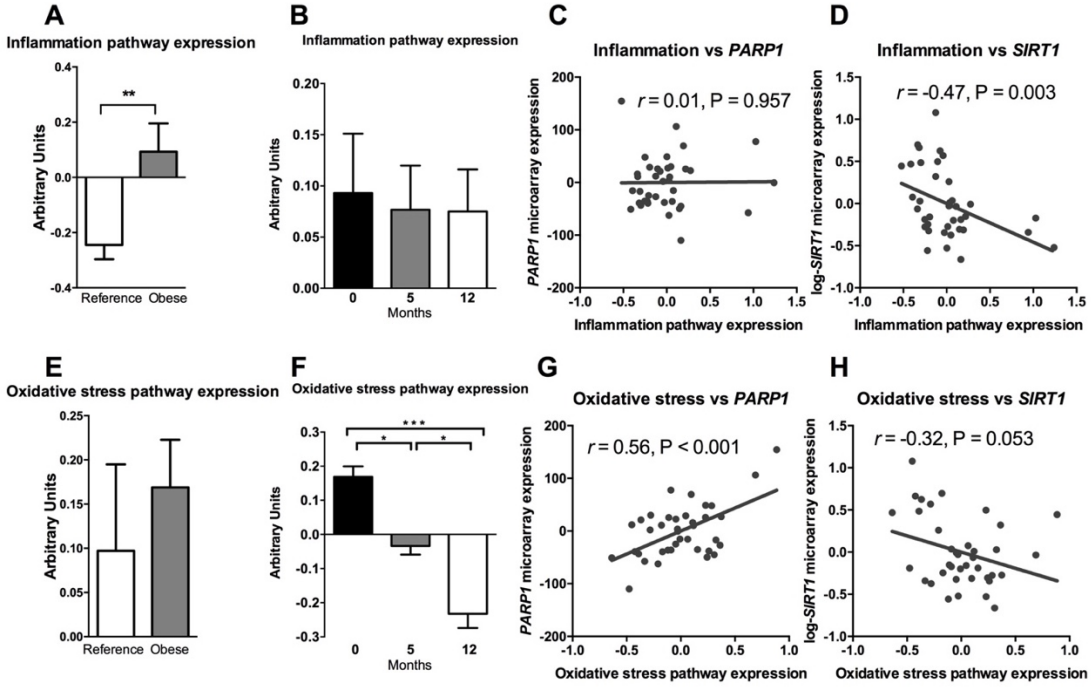

Figure 22 Inflammation- and oxidative stress-related gene expression pathways in the studied groups and their associations with *PARP1* and *SIRT1* expression. Mean centroid values of pathways depicting inflammation-related gene expression (A-D) and oxidative stress-related gene expression (E-H) from microarray data in SAT of the lean reference subjects (n = 19) and obese subjects (n = 19) are given. Figures apart from (B,C) are from baseline values. See Methods section 4.2.5 for how the mean centroid values were calculated and for the lists of genes included. Data are presented as mean ± SE. *P<0.05, **P<0.01, ***P<0.001. P-values were calculated with unpaired *t*-tests (A, E), Wald tests as post hoc tests for repeated measures ANOVA (B, F), or Pearson's correlation (C, D, G, H). The solid lines correspond to the linear least squares fit. Adapted from Rappou et al.[270].

5.3 CONTRIBUTIONS OF CARDIORESPIRATORY FITNESS AND ADIPOSITY TO METABOLIC HEALTH (STUDY III)

In Study III, two samples of twins were evaluated: Danish twins from the GEMINAKAR cohort and Finnish twins from the TwinFat sample (Table 8). We ran regression models on individual twins where various metabolic health variables were predicted with fat mass index (FMI = fat mass/height^2 [kg/m^2]), fat-free mass index (FFMI = fat-free mass/height^2 [kg/m^2]), and cardiorespiratory fitness (VO_{2max}/FFM [mL/(kg × min)]), with age and sex as additional covariates. The estimates from the two samples were aggregated with meta-analytic methods, except for VAT and liver fat %, which had only been measured in a subset of individuals from the TwinFat sample. In individuals, FMI was significantly positively associated with fasting glucose,

insulin resistance (HOMA-IR), acute insulin response (BIGTT-AIR), metabolic syndrome risk score (MetS score), systolic and diastolic blood pressure, LDL cholesterol, triglycerides, VAT amount, and liver fat % (Table 9). FMI was significantly negatively associated with insulin sensitivity (BIGTT-SI), and HDL cholesterol. The strongest of these associations were with HOMA-IR (β = 0.67), BIGTT-SI (β = -0.79), BIGTT-AIR (β = 0.53), MetS score (β = 0.69), VAT amount (β = 0.75), and liver fat % (β = 0.53) (Table 9). Other associations with FMI were weaker, but nevertheless significant ($|\beta|$ from 0.23 to 0.34). Regarding FFMI, effect sizes were weak and mostly not significant ($|\beta|$ from 0.00 to 0.13). Regarding VO_{2max}/FFM, the associations were also weak and mostly not significant ($|\beta|$ from 0.00 to 0.16) (Table 9).

Table 8. *Study III sample characteristics for GEMINAKAR and TwinFat.*

	GEMINAKAR		TwinFat		
	Female	**Male**	**Female**	**Male**	**Range**
Number of subjects	530	466	148	161	
% MZ	40	39	51	47	
Age (years)	37.4 (10.7)	37.1 (11.4)	28.2 (3.4)	28.8 (2.8)	18 - 63
BMI (kg/m^2)	23.9 (3.7)	24.7 (3.1)	25.5 (5.6)	25.7 (4.2)	16.3 - 48.6
Waist circumference (cm)	78.7 (9.5)	88.8 (8.7)	84.1 (13.1)	91.4 (11.9)	58.0 - 137.2
Fat mass (kg)	20.3 (7.9)	17.3 (6.7)	26.5 (12.7)	20.8 (10.8)	2.6 - 71.9
Fat free mass (kg)	46.2 (4.3)	62.4 (5.8)	43.9 (5.9)	62.3 (7.3)	31.3 - 84.1
Visceral adipose tissue (cm^3) (*n*=83)			780 (642)	1484 (1184)	95 - 5878
Liver fat % (*n*=83)			1.6 (2.2)	4.2 (5.5)	0.1 - 24
VO_{2max}/FFM (mL/(min × kg))	35.1 (7.7)	41.3 (8.4)	47.3 (7.4)	49.0 (8.3)	17.1 - 82.5
Fasting glucose (mmol/L)	4.70 (0.52)	4.90 (0.58)	4.99 (0.45)	5.22 (0.50)	2.4 - 8.3
Fasting insulin (pmol/L)	38.1 (19.9)	36.4 (19.6)	41.2 (23.8)	42.9 (26.9)	7.0 - 182.0
BIGTT-SI (AU)	11.79 (3.84)	10.58 (3.25)	10.60 (4.98)	9.20 (4.05)	1.30 - 23.44
BIGTT-AIR (AU)	2242 (1164)	2325 (1198)	2429 (1363)	2464 (1100)	658 - 13314
HOMA-IR (AU)	1.15 (0.64)	1.14 (0.61)	1.31 (0.75)	1.42 (0.91)	0.23 - 5.00
Systolic blood pressure (mmHg)	113.5 (12.6)	119.9 (12.6)	119.2 (9.5)	128.8 (10.4)	79 - 179
Diastolic blood pressure (mmHg)	67.9 (9.5)	70.2 (10.1)	74.4 (6.4)	78.7 (8.1)	44 - 108
HDL (mmol/L)	1.67 (0.43)	1.42 (0.41)	1.76 (0.46)	1.43 (0.35)	0.6 - 4.6
LDL (mmol/L)	3.22 (0.99)	3.36 (1.12)	2.32 (0.81)	2.67 (0.78)	0.2 - 7.2
Triglycerides (mmol/L)	1.21 (0.53)	1.40 (0.79)	1.03 (0.67)	1.16 (0.69)	0.2 - 5.5
Metabolic syndrome score (AU)	-0.10 (3.28)	-0.06 (3.54)	-0.28 (3.53)	-0.10 (4.08)	-8.61 - 11.21

Values are presented as "mean (SD)" and range "min - max". AU, arbitrary units; BIGTT-AIR, BIGTT acute insulin response index; BIGTT-SI, BIGTT insulin sensitivity index; FFMI, fat-free mass index (= fat-free mass / height^2 [kg / m^2]); FMI, fat mass index (= fat mass / height^2 [kg / m^2]); HDL, high-density lipoprotein; HOMA-IR, Homeostatic model assessment insulin resistance index; LDL, low-density lipoprotein; VO_{2max}/FFM (= VO_{2max} / fat-free mass [mL / (min × kg)]). Adapted from Jukarainen et. al.[290].

Table 9. *Linear multiple regressions of metabolic health variables by fat mass index (FMI), fat-free mass index (FFMI), and VO_{2max}/FFM. Combined results from both datasets.*

	Outcome variable	Predictor	ß	95% CI	P	R^2	N
Insulin action	Fasting glucose	FMI	0.30	(0.16, 0.45)	<0.001		
		FFMI	-0.06	(-0.22, 0.10)	0.445	0.13	1212
		VO_{2max}/FFM	0.03	(-0.03, 0.10)	0.292		
	HOMA-IR	FMI	0.67	(0.48, 0.86)	<0.001		
		FFMI	-0.11	(-0.22, 0.00)	0.053	0.27	1114
		VO_{2max}/FFM	-0.15	(-0.21, -0.09)	<0.001		
	BIGTT-SI	FMI	-0.79	(-0.89, -0.68)	<0.001		
		FFMI	0.03	(-0.06, 0.13)	0.505	0.50	1092
		VO_{2max}/FFM	0.10	(0.04, 0.17)	0.002		
	BIGTT-AIR	FMI	0.53	(0.41, 0.65)	<0.001		
		FFMI	0.11	(-0.01, 0.22)	0.071	0.25	1092
		VO_{2max}/FFM	-0.05	(-0.11, 0.01)	0.080		
Metabolic syndrome score		FMI	0.69	(0.59, 0.79)	<0.001		
		FFMI	0.10	(-0.11, 0.30)	0.354	0.46	1118
		VO_{2max}/FFM	-0.09	(-0.20, 0.02)	0.125		
Blood pressure	Systolic	FMI	0.32	(0.24, 0.40)	<0.001		
		FFMI	-0.01	(-0.12, 0.10)	0.880	0.26	1174
		VO_{2max}/FFM	-0.01	(-0.07, 0.05)	0.791		
	Diastolic	FMI	0.34	(0.26, 0.43)	<0.001		
		FFMI	0.00	(-0.15, 0.15)	0.998	0.25	1174
		VO_{2max}/FFM	0.05	(-0.01, 0.11)	0.081		
Lipids	LDL	FMI	0.27	(0.17, 0.36)	<0.001		
		FFMI	-0.02	(-0.25, 0.22)	0.894	0.21	1166
		VO_{2max}/FFM	0.00	(-0.16, 0.16)	0.987		
	HDL	FMI	-0.23	(-0.41, -0.05)	0.014		
		FFMI	-0.13	(-0.35, 0.09)	0.241	0.20	1190
		VO_{2max}/FFM	0.10	(-0.01, 0.20)	0.065		
	Triglycerides	FMI	0.32	(0.22, 0.43)	<0.001		
		FFMI	-0.03	(-0.15, 0.10)	0.694	0.12	1184
		VO_{2max}/FFM	-0.12	(-0.30, 0.06)	0.175		
Ectopic fat	Visceral adipose tissue volume[a]	FMI	0.75	(0.58, 0.86)	<0.001		
		FFMI	-0.05	(-0.25, 0.15)	0.603	0.61	83
		VO_{2max}/FFM	0.10	(-0.06, 0.25)	0.189		
	Liver fat %[a]	FMI	0.53	(0.31, 0.75)	<0.001		
		FFMI	-0.05	(-0.26, 0.21)	0.878	0.33	83
		VO_{2max}/FFM	0.16	(-0.04, 0.36)	0.799		

[a]Subjects only from TwinFat. All models include age and sex as covariates (coefficients not displayed). ß stands for standardized regression coefficients. BIGTT-AIR, BIGTT acute insulin response index; BIGTT-SI, BIGTT insulin sensitivity index; FFMI, fat-free mass index (= fat-free mass / height^2 [kg / m^2]); FMI, fat mass index (= fat mass / height^2 [kg / m^2]); HDL, high-density lipoprotein; HOMA-IR, Homeostatic model assessment insulin resistance index; LDL, low-density lipoprotein; VO_{2max}/FFM (= VO_{2max} / fat-free mass [mL / (min × kg)]). Adapted from Jukarainen et. al.[290].

Additionally, we repeated the above analysis by looking at the associations between intrapair differences (Δ) in MZ twin pairs, which controls for genetic and environmental factors shared within MZ pairs (Table 10). Regarding FMI, all of the associations from the individual-level analyses remained significant in this analysis with similar effect sizes, except for ΔHDL and Δtriglycerides. Regarding FFMI, the effects were again weak and mostly not significant (|β|

from 0.01 to 0.19). Regarding VO_{2max}/FFM, the effects were also weak and mostly not significant (|β| from 0.02 to 0.16) (Table 10).

Table 10. *Bootstrapped linear multiple regressions of intrapair differences (Δ) in metabolic health variables by intrapair differences in fat mass index (FMI), fat-free mass index (FFMI), and VO_{2max}/FFM.*

	Outcome variable	Predictor	ß	95% CI	P	R^2	N
Insulin action	ΔFasting glucose	ΔFMI	0.20	(0.01, 0.38)	0.042	0.09	253
		ΔFFMI	0.05	(-0.35, 0.45)	0.816		
		ΔVO_{2max}/FFM	-0.02	(-0.18, 0.14)	0.820		
	ΔHOMA-IR	ΔFMI	0.59	(0.22, 0.96)	0.002	0.28	229
		ΔFFMI	-0.13	(-0.42, 0.17)	0.405		
		ΔVO_{2max}/FFM	-0.16	(-0.33, 0.02)	0.084		
	ΔBIGTT-SI	ΔFMI	-0.68	(-0.87, -0.49)	<0.001	0.47	224
		ΔFFMI	0.05	(-0.13, 0.24)	0.578		
		ΔVO_{2max}/FFM	0.17	(-0.01, 0.36)	0.068		
	ΔBIGTT-AIR	ΔFMI	0.48	(0.18, 0.77)	0.001	0.25	224
		ΔFFMI	0.01	(-0.15, 0.17)	0.947		
		ΔVO_{2max}/FFM	-0.08	(-0.21, 0.04)	0.196		
ΔMetabolic syndrome score		ΔFMI	0.55	(0.40, 0.70)	<0.001	0.39	215
		ΔFFMI	0.08	(-0.08, 0.24)	0.333		
		ΔVO_{2max}/FFM	-0.09	(-0.19, 0.01)	0.083		
Blood pressure	ΔSystolic	ΔFMI	0.36	(0.15, 0.57)	0.001	0.13	231
		ΔFFMI	-0.04	(-0.23, 0.16)	0.718		
		ΔVO_{2max}/FFM	0.04	(-0.10, 0.17)	0.608		
	ΔDiastolic	ΔFMI	0.40	(0.20, 0.59)	<0.001	0.13	231
		ΔFFMI	-0.07	(-0.34, 0.20)	0.604		
		ΔVO_{2max}/FFM	0.04	(-0.10, 0.19)	0.554		
Lipids	ΔLDL	ΔFMI	0.47	(0.28, 0.67)	<0.001	0.16	243
		ΔFFMI	-0.18	(-0.40, 0.04)	0.103		
		ΔVO_{2max}/FFM	-0.10	(-0.23, 0.03)	0.119		
	ΔHDL	ΔFMI	-0.23	(-0.60, 0.13)	0.205	0.14	249
		ΔFFMI	-0.12	(-0.30, 0.06)	0.197		
		ΔVO_{2max}/FFM	0.13	(0.03, 0.24)	0.016		
	ΔTriglycerides	ΔFMI	0.30	(-0.03, 0.63)	0.077	0.11	245
		ΔFFMI	0.02	(-0.14, 0.18)	0.834		
		ΔVO_{2max}/FFM	-0.04	(-0.23, 0.16)	0.718		
Ectopic fat	ΔVisceral adipose tissue volume	ΔFMI	0.98	(0.86, 1.09)	<0.001	0.85	41[a]
		ΔFFMI	-0.13	(-0.27, 0.04)	0.439		
		ΔVO_{2max}/FFM	-0.05	(-0.21, 0.13)	0.764		
	ΔLiver fat %	ΔFMI	0.77	(0.52, 0.98)	<0.001	0.58	41[a]
		ΔFFMI	-0.11	(-0.35, 0.17)	0.504		
		ΔVO_{2max}/FFM	-0.16	(-0.41, 0.12)	0.335		

[a]Subjects only from TwinFat. ß stands for standardized regression coefficients. BIGTT-AIR, BIGTT acute insulin response index; BIGTT-SI, BIGTT insulin sensitivity index; FFMI, fat-free mass index (= fat-free mass / height^2 [kg / m^2]); FMI, fat mass index (= fat mass / height^2 [kg / m^2]); HDL, high-density lipoprotein; HOMA-IR, Homeostatic model assessment insulin resistance index; LDL, low-density lipoprotein; VO_{2max}/FFM (= VO_{2max} / fat-free mass [mL / (min × kg)]). Adapted from Jukarainen et. al.[290].

As reviewed in the introduction, different methods for scaling VO_{2max} result in different associations between CRF, as defined, and adiposity or variables associated with adiposity (such as insulin sensitivity). This is demonstrated in Figure 23 in the data for Study III. VO_{2max}/weight is strongly negatively correlated with FMI (Figure 23A-B, r = -0.46 for GEMINAKAR and r = -0.72 for TwinFat), whereas the allometric scaling method VO_{2max}/weight$^{0.67}$ makes

this association weaker (Figure 23C-D, r = -0.27 for GEMINAKAR and r = -0.59 for TwinFat). Scaling VO_{2max} with FFM resulted in the weakest associations between CRF and FMI (Figure 23E-F, r = -0.15 for GEMINAKAR and r = -0.00 for TwinFat).

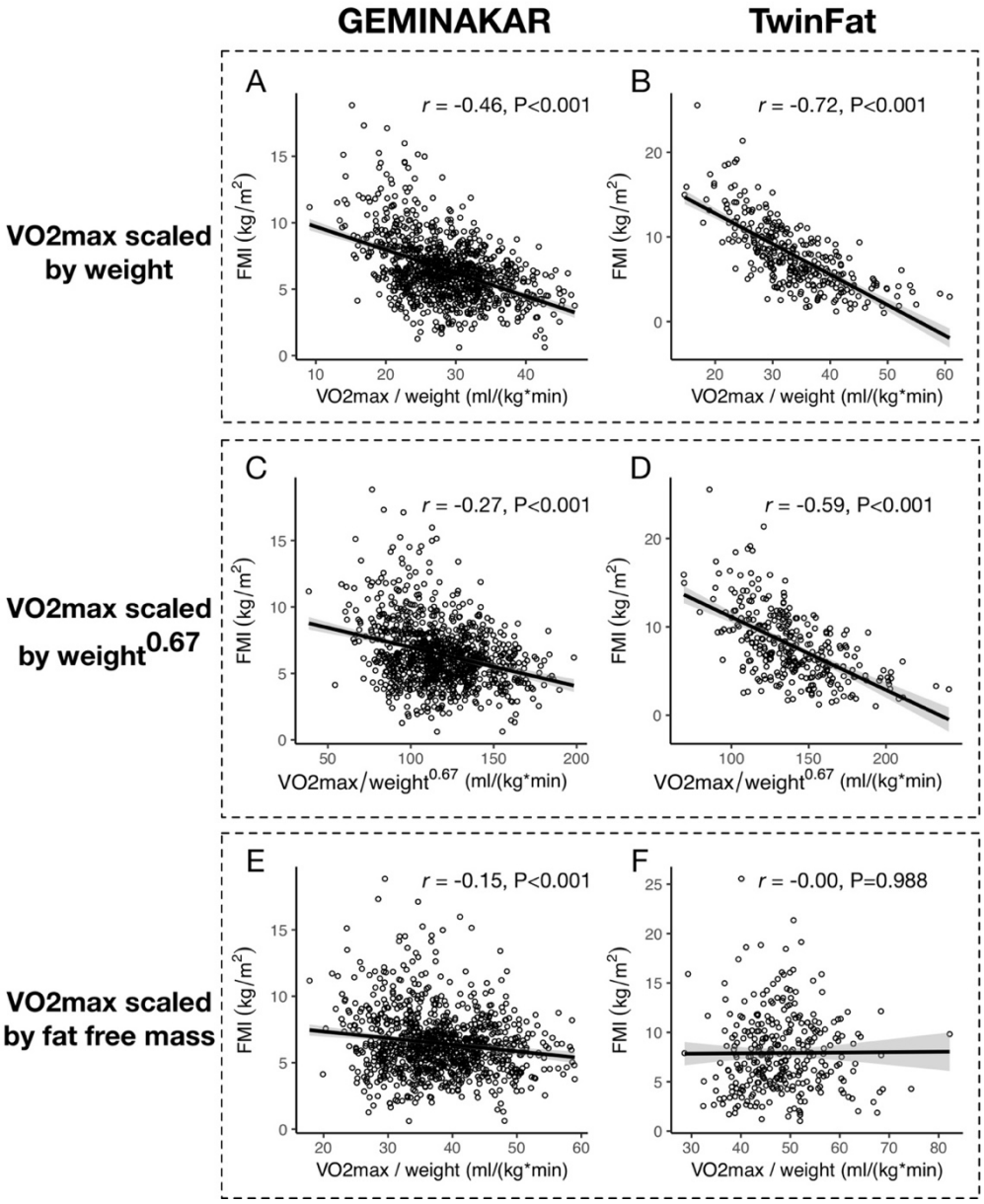

Figure 23 Relationships of VO_{2max} scaled by weight, weight$^{0.67}$, and fat-free mass with fat mass index in GEMINAKAR (n = 995) and TwinFat (n = 309) separately. Partial regression plots of VO_{2max}/weight and FMI (A-B), VO_{2max}/weight$^{0.67}$, and FMI (C-D), and VO_{2max}/FFM and FMI (E-F) after controlling for age and sex. Plotted values are the sum of individual residuals, after regressing them with age and sex, and the group mean. The effect sizes (r) and P-values correspond to Pearson's correlation coefficients. The black solid line represents the linear least squares fit. The shaded area corresponds to the 95% confidence interval of the fitted line. Adapted from Jukarainen et. al.[290].

To demonstrate how trying to control for the effect of body composition by stratifying individuals into BMI and CRF classes does not adequately control for body composition we grouped the individuals in GEMINAKAR into 1) groups of normal weight, with BMIs from 18.5 to 25 kg/m², and groups of overweight, with BMIs from 25 to 30 kg/m². We then calculated the sex-specific medians of VO_{2max}/weight and assigned each individual as being "unfit" (VO_{2max}/weight below median) or "fit" (VO_{2max}/weight above or at median). The mean fat % of normal-weight and overweight individuals stratified further as fit or unfit can be seen in Figure 24 (unpublished results), which demonstrates that this procedure leads to significant difference in group fat % between the fitness categories even after stratifying subjects by BMI ($P < 0.001$). This demonstrates how residual confounding can be a problem when trying to control for the effects of adiposity by stratifying subjects into groups by adiposity level, if the CRF measure is associated with adiposity (VO_{2max}/weight). The same logic should apply to CRF measured by weight-bearing exercise tests.

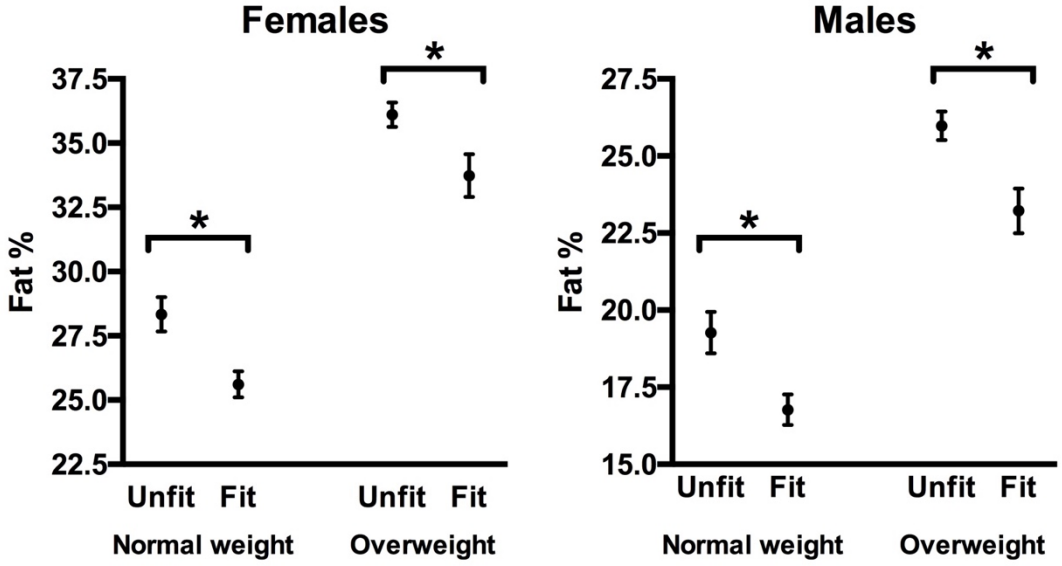

Figure 24 Comparison of the mean fat % between different VO_{2max}/weight categories within different strata of BMI in GEMINAKAR. "Normal weight" corresponds to BMI from 18.5 to 25 kg/m^2, "Overweight" corresponds to BMI 25 to 30 kg/m^2, "Unfit" corresponds to the lower half of sex-specific VO_{2max}/weight values, "Fit" corresponds to the upper half. Dots denote the group mean, bars denote the 95% confidence interval of the mean. *n* = 49-244 in each group. BMIs below 18.5 or over 30 kg/m^2 are not plotted due to lack of subjects in the respective categories of fit/unfit. Comparisons made with unpaired *t*-tests. *P < 0.001. Unpublished results.

6 DISCUSSION

6.1 SUMMARY OF MAIN FINDINGS

The most important results from Study I are the following: The expressions of *SIRT1*, *SIRT3,* and *SIRT5* and NAD^+ biosynthesis genes in SAT are downregulated in heavier co-twins of BMI-discordant pairs, indicating that acquired or unique environmental differences related to obesity affect the expressions of these genes in SAT. We also demonstrated that in MZ twins there are negative correlations between intrapair differences in SAT *SIRT1*, *SIRT3*, *NAMPT*, *NMNAT3,* and *NRK1* expressions and various adiposity measures. There are also negative correlations between MZ intrapair differences in SAT *SIRT1*, *SIRT5*, *NMNAT2*, *NMNAT3* and *NRK1* expressions and measures of SAT inflammation. Also, MZ intrapair differences in *SIRT1* and SIRT5 are negatively correlated with insulin resistance measures. These results indicate that there are unique environmental correlations between the the abovementioned variables, and the individual (or phenotypic) correlations between these variables are not only due to genetic or shared environmental confounding. Finally, some genes associated with the SIRT1 downstream pathway UPR^{mt} were expressed at significantly lower levels in the SAT of the heavier co-twins of the BMI-discordant MZ twin pairs, possibly due to lowered SIRT1 activity due to acquired obesity.

The major findings from Study II are summarized next. The baseline results show that the SAT expressions of *SIRT1*, *SIRT3*, *SIRT7*, and *NAMPT* are significantly lower in obese (but otherwise healthy) subjects than in leaner reference subjects. Additionally, in all subjects body fat % was correlated negatively with expression of SAT *SIRT1* and positively with total SAT PARP activity. The longitudinal results from the weight loss intervention further showed that *SIRT1* expression increased with concomitant weight loss from 0 to 5 months in all subjects, but reverted to previous levels in subjects who had regained some of the weight between 5 and 12 months. SAT total PARP activity also decreased and *NAMPT* expression increased during weight loss. Despite the baseline correlations between adiposity and *SIRT1* or *NAMPT* expression, and the observed effect of the weight loss intervention on *SIRT1* and *NAMPT* expression, the correlations between longitudinal change in fat % or liver fat % and longitudinal change in *SIRT1* or *NAMPT* expression were not significant. In summary, the results from Study II suggest that SAT in humans responds to reduced caloric intake, increased physical activity, and/or weight loss by increasing NAD^+/SIRT pathway expression with a concomitant decrease in total PARP activity, possibly promoting metabolic health through these mechanisms.

The main results from Study III show that adiposity (fat mass index, FMI) is significantly negatively associated with insulin sensitivity and positively

associated with fasting glucose, insulin resistance, blood pressure, LDL cholesterol, triglycerides, metabolic syndrome risk, VAT amount, and liver fat %, even after controlling for genetic and shared environmental factors by looking at the associations of MZ twin intrapair differences. This points to a unique environmental association between adiposity and the abovementioned metabolic health variables. While this is not direct evidence for causal associations, the presence of these unique environmental correlations outlines the opportunities for environmental modifications of the variables because genetic and shared environmental factors are controlled for in the approach. Conversely, and perhaps surprisingly, the associations between cardiorespiratory fitness (VO_{2max}/FFM) and these metabolic health variables were weak or nonsignificant (with $|\beta| \leq 0.17$). Similarly, associations of fat-free mass (FFM) with the outcome variables were weak and mostly nonsignificant. These results imply that when CRF is measured in a way that is not confounded by adiposity (VO_{2max}/FFM) CRF is only very weakly associated with metabolic health, underlining the relative importance of adiposity.

6.2 EVIDENCE FOR OBESITY AFFECTING THE NAD$^+$/SIRT PATHWAY

In Study II, we showed that obese weight loss intervention participants had lower SAT *SIRT1*, *SIRT3*, and *SIRT7* expression than lean reference subjects, with no significant difference in *SIRT2* or *SIRT5* expression. In Study I, evaluating a sample of young adult MZ twins discordant for BMI, we showed that the SAT expressions of *SIRT1*, *SIRT3*, and *SIRT5* are downregulated in the heavier co-twins, with no significant differences in *SIRT2* or *SIRT7*. Because by definition, any differences within MZ twin pairs must result from unique environmental differences between these twins, this is evidence for adiposity (or BMI) having a unique environmental association with SAT *SIRT1*, *SIRT3*, and *SIRT5* expressions. However, when analyzing the same BMI-discordant MZ twin pairs together with MZ twins not discordant for BMI, looking at the associations between intrapair differences in *SIRT* expression and adiposity, only *SIRT1* and *SIRT3* were significantly associated with adiposity variables (BMI, fat %, and VAT amount), with *SIRT1* having a markedly larger effect size (r from -0.76 to -0.85) than *SIRT3* (r from -0.34 to -0.40). Only *SIRT1* and *SIRT3* expressions were robustly associated with adiposity in both Studies I and II, whereas *SIRT5* and *SIRT7* expressions were associated in some of the analyses, but not in others.

Because SIRT activity is regulated by availability of NAD$^+$[291], we additionally analyzed the expression of NAD$^+$ biosynthesis genes, with NAMPT being the rate-limiting enzyme of NAD$^+$ synthesis through the salvage pathways[165]. We demonstrated that multiple genes involved in NAD$^+$ biosynthesis have higher expression in the heavier co-twins of BMI-discordant

pairs and that they correlate negatively with measures of adiposity in individuals and intrapair differences (*NAMPT*, *NMNAT2*, *NMNAT3*, *NRK1*). Furthermore, in 10 BMI-discordant pairs we demonstrated a P = 0.051 trend for higher total PARP activity, PARPs being the most important intracellular NAD^+ consumers, competing for the same NAD^+ pool with SIRTs[26], which have been shown to lower SIRT activity[168–170]. The downregulation of *NAMPT* expression and upregulation of total PARP activity in SAT due to obesity was replicated in Study II, comparing values from obese individuals with those from lean reference subjects. Furthermore, SAT total PARP activity was positively correlated with fat % at baseline in all subjects of Study II, and there was a significant decline in SAT total PARP activity after weight loss from 0 to 12 months in the intervention group.

Previous evidence in humans for the effect of obesity on SAT sirtuin expression comes from studies showing cross-sectional associations between BMI and *SIRT1* expression[158–162], *SIRT3* expression[160], and *SIRT6* expression[160]. SAT *NAMPT* expression has also been observed to be negatively associated with BMI or obesity[166,167]. These studies might, however, be confounded by common genetic factors or early environmental factors influencing *SIRT* or *NAMPT* expression and adiposity. Our results with a genetically informative twin sample confirm that there indeed are unique environmental associations between BMI (or adiposity) and SAT *SIRT1*, *SIRT3*, and *NAMPT* expressions. *SIRT6* expression was not, however, detectable by our microarray.

Additionally, the association in individuals between SAT *SIRT1* expression and BMI was somewhat stronger (r = -0.63) than previous studies have reported (r from -0.32 to -0.44)[158,160,162]. This might be explained in at least three ways: 1) different degrees of measurement error, 2) different degrees of mean obesity in the studied subjects, 3) different degrees of heterogeneity in obesity in the studied subjects. Regarding explanation 1: Moschen et al.[160] and Song et al.[292] determined SAT *SIRT1* expression with quantitative RT-PCR; a possible higher measurement error than with the microarray that we used could contribute to weaker associations. Regarding explanation 2: their subjects were more obese (with BMIs of 43.2 kg/m^2[160] and 29.3 kg/m^2[292]) than ours (27.8 kg/m^2), which could weaken the association if there is a floor effect of BMI on SAT *SIRT1* expression at higher BMIs. We indeed observed that the association between BMI and *SIRT1* expression may be curved and approximately log-linear, flattening out at BMI > 30 kg/m^2 (Figure 12). However, reliable modeling of this kind of a nonlinear association requires larger sample sizes at the extremes, and thus, this observation is merely preliminary. In support of this explanation, Clark et al. [158], who also measured SIRT1 expression with a microarray, observed that the association between BMI and SAT *SIRT1* expression is stronger at r = -0.36 in a group with lower BMIs (mean around 23 kg/m^2) than in a group with higher BMIs (mean around 39 kg/m^2) at r = -0.11. Why the correlations in Clark et al.[158] were lower even in the low BMI group than in our study is, however, not clear. 3) Finally,

a higher degree of heterogeneity in obesity or BMI in the TwinFat sample due to discordance sampling might also inflate the observed associations in Study I compared to other studies with more random sampling.

Alterations in SAT *SIRT* and NAD^+ biosynthesis gene expression probably reflect the influence of environmental factors such as excessive caloric intake or sedentary behavior. There is indeed evidence that long-term high-fat feeding decreases the expression, protein levels, and activity of *Sirt1* and causes proteolytic cleavage of SIRT1 by Caspase-1 in mouse SAT[157]. High-fat feeding has also been demonstrated to increase *Sirt3* expression in hepatic and muscle tissue of mice[293,294]. Regarding the NAD^+ side, high-fat feeding has been shown to decrease cellular NAD^+ levels in mouse SAT due to the downregulation of *Nampt* expression[295] and to increase PARP activity in mouse muscle[168]. Combined with the observation that increasing NAD^+ levels increases *Sirt1* expression at least in mouse liver tissue[155], these studies suggest that the observed downregulation of *SIRT1* and *SIRT3* in heavier co-twins could, at least partially, follow from reduced NAD^+ availability. This reduction in NAD^+ availability could be the result of reduced synthesis because of lowered *NAMPT* expression and increased consumption due to increased PARP activity.

While we cannot specify the unique environmental differences within twin pairs resulting in differences in adiposity and NAD^+/SIRT pathway activity, differences in diet and physical activity are the most plausible candidates. Indeed, the heavier co-twins of BMI-discordant pairs did report significantly less physical activity. However, we did not find differences in the food record data between the BMI-discordant co-twins, but based on an earlier study of the twins, using doubly-labeled water for determining energy consumption, the heavier co-twins underestimated their food intake and overestimated their physical activity level[296]. Thus, we probably do not have exact measures of food intake and physical activity that can be used to reliably test these explanations.

6.3 NAD^+/SIRT PATHWAY AND WEIGHT LOSS

By following subjects involved in a weight loss intervention in Study II, we observed that weight loss from 0 to 5 months elevated the SAT expression of *SIRT1*. There are at least two previous studies in humans investigating the effects of weight loss or caloric restriction on *SIRT* expression. Pedersen et al.[161] evaluated 9 obese nondiabetic women with BMI of around 46 kg/m^2 fasting for 6 days. *SIRT1* expression from isolated SAT adipocytes was significantly increased after 6 days of total fasting relative to baseline. Moschen et al.[160] studied 29 subjects with BMI of around 43 kg/m^2 undergoing laparoscopic adjustable gastric banding before the procedure and 6 months after the procedure. SAT *SIRT1*, *SIRT3*, and *SIRT6* expressions were significantly upregulated at 6 months after the procedure compared with baseline. Our results complement these two studies and show that a weight

loss intervention increases SAT *SIRT1* expression even in non-morbidly obese subjects with BMIs of around 35 kg/m², even without drastic measures such as laparoscopic adjustable gastric banding as in Moschen et al.[160] or total fasting as in Pedersen et al.[161]. Additionally, there is at least one human study showing that caloric restriction and/or increased physical activity increases *SIRT1* expression in human muscle[297]. Complementing these results for humans, results from animal studies have shown that *Sirt1* expression is elevated by caloric restriction in various tissues, including adipose tissue[156].

Along with increased SAT *SIRT1* expression, we demonstrated changes related to possible increased NAD^+ availability in SAT resulting from the weight loss intervention. We showed that SAT *NAMPT* (the rate-limiting enzyme in NAD^+ synthesis through the salvage pathways[165]) expression was increased, whereas the activity of PARPs (important consumers of cellular NAD^+) decreased along with reduced *PARP1* expression levels at the end of the weight loss intervention compared with baseline. To my knowledge, there are only animal studies assessing the effects of caloric restriction on SAT *NAMPT* and *PARP* expression and total PARP activity. Caloric restriction has been shown to increase NAD^+ levels and the NAD^+/NADH ratio in mouse SAT[130] and to increase *Nampt* expression at least in mouse skeletal muscle cells and cultured C2CL12 skeletal muscle cells [131,298]. There is also indirect evidence for reduced SAT PARP expression and activity due to fasting. Bai et al.[168] have demonstrated that a 24-h fast reduces total PARP activity in mouse muscle cells, whereas high-fat feeding increases *Parp-1* expression and PARP activity measured as PARP-1 autoPARylation levels. Additionally, they demonstrate that compared with wild-type mice, *Parp-1* knockout mice develop more brown adipose tissue in which there is lower PARP activity, higher NAD^+ content, higher SIRT1 content, and higher FOXO1 acetylation, indicative of higher SIRT1 activity[168]. Incubating mouse hepatocytes (Hepal-6) in a NAD^+ rich medium increases *Sirt1* expression[155], suggesting that increased NAD^+ availability might not only increase SIRT1 activity but also its expression. Taken together, these studies on rodents or cell cultures suggest the following interpretation of our results: the weight loss intervention increased *NAMPT* expression and decreased *PARP-1* expression and total PARP activity, all of which lead to an increase in cellular NAD^+ availability, and thus, increased *SIRT1* transcription and possibly SIRT1 activity (although SIRT1 activity was not assessed directly). A similar interpretation can be made to explain the results of Study I on twins since the unique environmental differences between the MZ twins are probably a combination of differences in caloric intake, fat intake, and physical activity.

In a way, the results from Study I on MZ twin pairs and the results from Study II on weight loss study participants can answer very similar questions, even though one is a cross-sectional twin study and the other a longitudinal study on unrelated individuals. In Study II, we can see the effect of a change in environment (weight loss intervention) while keeping genes and the earlier environment fixed (by studying the same individual at different time points).

Similarly, in Study I, with MZ twin pairs discordant for BMI, we can see the effect of the long-term (unique) environment, leading to a BMI difference, while keeping genes and the shared environment fixed (by studying MZ twin pairs sharing genetic and shared environmental factors). The main advantages of a longitudinal intervention study are that the environmental change can be specified, and if it is a randomized controlled trial, the causal effect of the intervention can be assessed more directly. While in cross-sectional twin studies, such as Studies I and III, one cannot directly specify or manipulate the environmental factors of interest, the main advantage is that the effects of long-term environmental factors are present in the associations.

For example, if individuals in a MZ pair are discordant in BMI, one can think of the heavier co-twin as an answer to the counterfactual "what if the leaner twin had lived in an environment that made the individual gain more weight". So MZ twin pairs discordant in some aspect are in essence natural experiments indicating what would have happened to an individual (keeping genes and shared environment fixed) had he or she lived in a different (unique) environment that produces the observed discordance. Continuing the example, if MZ pairs selected to be discordant for BMI are also discordant in another feature, e.g. SAT *SIRT1* expression, SAT *SIRT1* expression must have unique environmental causes that are common with those of BMI, in other words, SAT *SIRT1* expression and BMI have a unique environmental correlation (r_E). This is different from just demonstrating a phenotypic association between BMI and SAT *SIRT1* expression in unrelated individuals since a phenotypic association can theoretically consist of any combination of genetic (A), shared environmental (C), and unique environmental (E) correlations: $r_P = r_A + r_C + r_E$. However, observing a unique environmental correlation (r_E) between BMI and *SIRT1* expression does not imply that raising BMI lowers *SIRT1* expression, or vice versa, since any number of unobserved variables could affect both of them. Possibly only experimental mechanistic studies or pseudo-experimental studies, such as Mendelian randomization studies[35], can reveal the more precise causal structure behind the observed correlations.

In summary, our results from Studies I and II suggest that the lowering of expression of *SIRT*s and NAD^+ biosynthesis genes and the induction of PARP activity represent early metabolic changes in SAT due to obesity or the long-term environmental factors leading to obesity (such as high caloric intake, a high-fat diet, or a sedentary lifestyle). These changes in the NAD^+/SIRT pathway are observable in healthy subjects without overt metabolic disease, indicating that alterations in the NAD^+/SIRT pathway might be factors involved early on in the pathogenesis of obesity-related disease such as T2DM. These changes seem to be reversible by lifestyle modifications, as SAT *SIRT1* expression increased and total PARP activity decreased after the weight loss intervention in Study II. The results support the notion that pharmacological interventions[129] affecting the NAD^+/SIRT pathway (NAD^+ boosters or SIRT

activators) or PARP inhibitors could be promising targets of drug development for metabolic disease.

6.4 NAD$^+$/SIRT PATHWAY IN OBESITY AND METABOLIC HEALTH

If obesity or weight loss affects the expression or the activity of the NAD$^+$/SIRT pathway in SAT, as the evidence from Studies I and II suggests, what is the possible relevance of SAT SIRT activity for metabolic health? As reviewed in the Introduction, multiple animal studies show that whole-body *SIRT1* overexpression or activation through sirtuin-activating compounds leads to more lean, insulin-sensitive, and glucose-tolerant phenotypes, and seems to provide protection from metabolic dysfunction caused by high-fat feeding[170,172,176–179,179]. Additionally, adipose tissue-specific overexpression of *SIRT1* in mice has been shown to lead to a similar beneficial phenotype[181], and in contrast, adipose tissue-specific *SIRT1* knockout[157,183] or knockdown[181] mice have more metabolically dysfunctional phenotypes. However, in one study adipose tissue-specific knockout mice on a high-fat diet were at first more insulin-resistant and glucose-intolerant than wild-type controls, but developed less metabolic dysfunction than the wild-type mice after long-term high-fat feeding[181].

Similarly, in humans, whole-body SIRT activation through STACs has been shown to reduce metabolic dysfunction in some studies. A meta-analysis of 6 small and heterogeneous randomized controlled clinical trials on resveratrol supplementation in individuals with T2DM showed significant reductions in HbA1c and systolic blood pressure, but no significant changes in HOMA-IR, diastolic blood pressure, fasting insulin, triglycerides, LDL, or HDL cholesterol[187]. One of the two phase II clinical trials on SRT2104 (a SIRT1-activating compound) that have been completed has been published. Its results do not show a clear dose-response effect on insulin sensitivity or glucose tolerance, although there was a significant weight reduction and modest improvements in lipid profiles[188]. It must be noted though that plasma SRT2014 concentrations were not dose-proportional and the target plasma levels were not achieved, and thus, problems with pharmacokinetics might explain the lack of effect. Additionally, two small phase I clinical trials on SRT2014 in healthy elderly subjects have observed significant decreases in body weight, LDL cholesterol, and triglyceride levels in the SRT2014 groups compared with placebo groups[189,190]. In summary, results on whole-body SIRT1 activation in humans by administering STACs do not unanimously show a benefit with respect to insulin sensitivity, glucose tolerance, or lipids.

Evidence linking SAT *SIRT1* expression or activity to metabolic health in humans is also scarce. While multiple studies have shown lowered SAT *SIRT1* expression to be linked to obesity[158–162], it is not clear whether this is reflected in increased SAT dysfunction. SAT *SIRT1* expression has been shown to be

positively associated with whole-body insulin sensitivity, SAT expression of PGC-1α (regulator of mitochondrial biogenesis), and other target genes regulated by SIRT1[186]. Similarly, three single-nucleotide polymorphisms of *SIRT1* have been shown to be associated with total energy expenditure during a hyperinsulinemic-euglycemic clamp test[178]. Our results in Study I, demonstrating a positive link between SAT *SIRT1* expression and multiple measures of insulin sensitivity, corroborate these findings. However, it is not immediately clear why low SIRT1 activity or expression in SAT would lead to systemic insulin resistance or glucose intolerance. This is because the physiology of skeletal muscle[184] and hepatic tissue[3] are the main factors in determining whole-body glucose metabolism. It could well be that some factors, such as nutrient excess, affect both insulin sensitivity in various tissues and SAT *SIRT1* expression, making their association non-causal or spurious. However, animal studies on SAT-specific *SIRT1* knockouts or knockdowns, showing alterations in whole-body insulin sensitivity and glucose tolerance[157,181,183], point to the existence of a causal mechanism.

SAT inflammation has been proposed to possibly lead to systemic metabolic dysfunction[2,17,18]. SIRT1, known to suppress inflammation at least by repressing NF-κB[137], has been shown to regulate SAT inflammation in animal studies[157,159]. Thus, SAT SIRT1 activity is a possible factor mediating the effects of obesity on adipose tissue inflammation (and subsequently whole-body metabolic dysfunction). In humans, SAT SIRT1 expression has been demonstrated to be negatively correlated with SAT expression of macrophage markers[159] and macrophage infiltration determined by immunohistochemistry[163]. Furthermore, individuals of equal BMI with more VAT have lower SAT SIRT1 expression, more SAT macrophage infiltration, and higher SAT inflammatory gene expression[163]. Our results corroborate these findings. In Study I, we demonstrated significant negative associations of SAT *SIRT1* and *SIRT5* expression with SAT expression of CD14 (a macrophage marker), SAT expression of multiple inflammatory pathways, and serum hs-CRP, even after controlling for genetic and shared environmental factors with the MZ twin intrapair differences method. Furthermore, the demonstrated negative association between *SIRT5* expression and inflammatory markers has, to my knowledge, not been reported previously. As SIRT5 is a mitochondrial sirtuin not known to regulate inflammation[146], it is probable that some unknown process associated with inflammation has led to the reduced expression of *SIRT5*. It must be noted that the individual-level associations between inflammation and SIRT1 were higher than the intrapair difference associations (significance not tested), which indicates that genetic or shared environmental factors account for a part of the stronger observed phenotypic association.

Additionally, we demonstrated that the heavier co-twins of the BMI-discordant MZ pairs had significantly lower expression of 4 of the studied 9 genes related to UPR^{mt} (Figure 15), a recently discovered downstream pathway of the NAD^+/SIRT1 pathway[144] that seems to promote mitochondrial protein

homeostasis in animal models[299]. Although the role of this UPR^{mt} pathway is still unclear in mammals, its existence or activation has been previously demonstrated in mouse[170] and human[300] muscle tissue. Our results suggest that the human UPR^{mt} pathway could possibly be downregulated in SAT due to obesity. This finding awaits further confirmation and its possible role in regulating mitochondrial health and function in mammals remains to be elucidated.

6.5 CARDIORESPIRATORY FITNESS AND METABOLIC HEALTH

The relative importance of CRF and adiposity in explaining the abnormalities associated with metabolic syndrome, T2DM, or other disease states connected to lifestyle factors has generated abundant discussion, and numerous studies have attempted to answer this question. The results from Study III clearly show that adiposity is strongly negatively associated with metabolic health. However, the associations between the studied metabolic health variables and CRF (VO_{2max}/FFM) appear to be very weak, even after controlling for genetic and shared environmental factors with the MZ twin intrapair differences approach. However our results are in conflict with many previous studies claiming moderate or strong associations between CRF and insulin sensitivity[240,243,244,246,250], metabolic syndrome subcomponents[257] or risk[258,259], ectopic fat accumulation[262,264], T2DM risk[14], cardiovascular disease outcomes[14], and mortality[15,232,233,233–236,238,239,267]. The problems with measuring CRF independently of adiposity reviewed previously in the introduction could explain why many studies find meaningful associations between CRF and metabolic health. To summarize, the problems in measuring CRF independently of adiposity arise from multiple possible sources:

1) Estimating CRF from performance in weight-bearing exercise tests, an approach used in many large-scale epidemiological studies on mortality[232,233,233–236,238,239], T2DM[14], and cardiovascular disease outcomes[14], which biases the measurement of CRF against more adipose subjects[227] could lead to adiposity confounding the association between CRF and outcomes, if the effect of adiposity is not properly controlled.

2) Even if VO_{2max} is measured directly or estimated in a way that does not underestimate VO_{2max} in more adipose individuals (e.g. bicycle ergometry), there can be a problem with confounding. If VO_{2max} is scaled to body size erroneously by dividing it with total body weight, for the reasons outlined in the Introduction, this biases the measure of CRF against more adipose individuals and does not appropriately control for body weight[211–217].

3) Even if a CRF measure is confounded by adiposity, the effect of adiposity can possibly be controlled by proper statistical techniques such as multivariate linear regression. However, many studies try to control for the effect of adiposity by stratifying subjects into categories of adiposity (e.g. BMI

categories), and look for the association between CRF and outcome variables within the strata of BMI[14,15,262,264]. I argue that this is generally not sufficient to control the effect of BMI on the measure of CRF since if the CRF measure is negatively correlated with adiposity or BMI, this will lead to residual confounding[202–205]. This was illustrated with our data for Study III (Figure 24). When the subjects in GEMINAKAR with BMI from 18.5 to 30 kg/m² are grouped by BMI into the following classes: normal weight with BMI from 18.5 to 25 kg/m² and overweight with BMI from 25 to 30 kg/m², and if they are also grouped by VO_{2max}/weight with a sex-specific median split into low CRF and high CRF groups, the low CRF groups have significantly higher fat % even within the BMI groups (Figure 24). This suggests that studies with a CRF measure that is associated with adiposity cannot fully control for the effect of adiposity in the association between the CRF measure and metabolic variables by stratifying subjects into groups according to body composition, as this leads to residual confounding[202–205].

One motivation behind Study III was to use a CRF measurement not confounded with adiposity (VO_{2max}/FFM) to examine the associations between CRF and metabolic health, independently of adiposity. A few previous studies that we know of have investigated the association of VO_{2max}/FFM with metabolic health and these studies give results somewhat similar results to ours. The two high-quality studies with direct VO_{2max} measurements of subjects aged 8-18 years have found either a very weak association ($|r|$ = 0.11)[218] or no significant association[248] between CRF and insulin sensitivity. The studies that have observed stronger associations between VO_{2max}/FFM and insulin sensitivity have smaller sample sizes and either estimate VO_{2max} indirectly from bicycle ergometry[245,249] or have methodological problems (calculating the correlation across highly heterogeneously sampled groups of subjects)[246].

Regarding components of metabolic syndrome (e.g. fasting glucose, HbA1c, HDL, total cholesterol, triglycerides, and blood pressure), large longitudinal studies looking at the change in CRF and outcome variables while controlling for the change in body composition have found very small independent effects of CRF (corresponding to $|r|$ of 0.00 to 0.10 or ΔR^2 of 0.0% to 1.1%)[260,261]. Results from these two studies agree with our cross-sectional estimates. There is one longitudinal study that investigated the effect of change in CRF, while controlling for change in VAT amount, which shows that change in CRF increase R^2 of the model by 6.5% (corresponding to a semipartial r = -0.25)[301]. The cross-sectional studies that provide negative associations between CRF and metabolic syndrome components[257] or the odds of metabolic syndrome diagnosis[258,259] have estimated CRF from performance in weight-bearing exercise tests[257], scaled VO_{2max} by dividing it with weight[258], or formed CRF groups by categorizing VO_{2max}/weight into groups by cutoff points[259]. One cross-sectional study looking at the association of VO_{2max}/FFM with metabolic syndrome components and a continuous metabolic syndrome score[218] has shown that VO_{2max}/FFM has weak and mostly nonsignificant associations with

total cholesterol, HDL cholesterol, triglycerides, mean blood pressure, and HOMA-IR (|*r*| from 0.02 to 0.18), and a weak association with a continuous metabolic syndrome score (r = -0.13) which becomes non-significant (r = -0.07) after adjustment by body fat, sex, height, and ancestry. Regarding VAT and liver fat accumulation, our results from the TwinFat cohort show no significant associations with VO_{2max}/FFM, but strong associations with FMI. However, we had limited statistical power to detect small effect sizes. We found no previous studies examining the association between ectopic fat accumulation and VO_{2max}/FFM, although at least two studies have claimed that there might be a connection [262,264], but they are probably confounded by adiposity as discussed in the introduction.

There seems to be conflict between the general belief that physical fitness is essential to metabolic health and our results demonstrating very weak associations between CRF and the studied metabolic health variables. However, this might not be as surprising as it seems at first glance. As reviewed in the introduction, differences in VO_{2max} between healthy individuals[196] and the changes in CRF within individuals due to endurance training[193,201] seem to be explained by differences or changes in maximal cardiac output (Q_{max}), or more precisely in maximal stroke volume (SV_{max}), as maximal heart rate (HR_{max}) does not vary or change much. SV_{max} is determined by left ventricular end diastolic volume (affected mainly by preload, which is determined from blood volume, venous return, and right ventricular function), left ventricular contractility, and total peripheral vascular resistance[195], which are factors not directly connected to tissues thought to determine metabolic health (e.g. skeletal muscle, adipose tissue, and liver). Thus, differences or changes in VO_{2max} seem to follow from factors not directly connected to metabolism, which makes the observed disconnection between VO_{2max}/FFM and metabolic health variables not that surprising or counterintuitive. This does not, however, mean that physical activity or exercise is not important for metabolic health. Only that VO_{2max} is perhaps not the factor mediating the beneficial effects of exercise on metabolic health. VO_{2max} should perhaps not be seen as a simple measure of general fitness, but should be viewed more as a measure of maximal cardiovascular function. Fitness, as it is perhaps defined in layman's terms, corresponds more to exercise capacity and/or being lean or relatively muscular, factors that are not really measured by VO_{2max}. Of course, our discussion of VO_{2max} applies to healthy individuals without overt heart, lung, or other kinds of disease affecting cardiorespiratory physiology. The usefulness of VO_{2max} in clinical physiology or cardiopulmonary disease patients is a separate discussion beyond the scope of this thesis.

There are some limitations with Study III that merit discussion. The indirect estimation of VO_{2max} from bicycle ergometry exercise test performance and the measurement of body composition with bioelectrical impedance analysis in GEMINAKAR are not gold standard measurements, whereas the spiroergometry and DEXA in TwinFat could be considered gold standard. Estimation of VO_{2max} with bicycle ergometry could in theory

underestimate fitness in more obese individuals, and a possible indication of this can be seen in Figure 23; for the TwinFat cohort, VO_{2max}/FFM had a near-zero partial correlation with FMI after adjusting for age and sex (r = -0.01), whereas in GEMINAKAR the same correlation was significant (r = -0.14, P<0.001, corresponding to a R^2 of 2.0%). While this is a minimal effect, it could come from more adipose individuals having relatively higher workloads during the exercise test due to the weight of the legs. It did not, however, lead to VO_{2max}/FFM being a meaningfully better predictor of outcome variables in GEMINAKAR than in TwinFat in the regression models reported.

Also, despite the aggregation of the estimates with meta-analytic methods, there was some statistically significant heterogeneity between the estimates from the two samples (see supplements of Study III). Despite this heterogeneity, we chose to report and discuss the aggregated effects since we do not have good justification for preferring the estimates from one sample to another. Also, we cannot reliably assess the origins of the heterogeneity. Even if measurements in TwinFat were more gold standard, the sampling procedure was less random because part of the sample was selected on the basis of high BMI discordance. Despite the heterogeneity, the most important conclusions from our study can be made even when considering both samples separately; in both samples, FMI was clearly the best predictor of the outcome variables, whereas FFMI and VO_{2max}/FFM had only very weak associations. In individual analyses, the largest discrepancies between estimates from the two samples were in the association between VO_{2max}/FFM and triglycerides (GEMINAKAR β = -0.21 and TwinFat β = -0.03). Additionally, in within-pair analyses for Δfasting glucose, βs for ΔFFMI in GEMINAKAR were -0.15 and in TwinFat 0.26.

Also, the total R^2s and βs for FMI tended to be higher in TwinFat both in the individual-level analyses and in intrapair analyses. This could reflect the fact that the subjects in TwinFat were on average slightly more obese and had more variation in BMI: average and standard deviation in BMI were 24.4 ± 3.5 kg/m² in GEMINAKAR and 25.7 ± 5.1 kg/m² in TwinFat. Especially in the within-pair analysis of MZ twin pairs, with 20 MZ pairs being sampled for high BMI discordance (ΔBMI > 3 kg/m²), the presence of higher than expected discordance in BMI probably to some extent inflates the estimates of associations between BMI-associated traits such as FMI and the metabolic health variables studied. Even though the sampling procedure in TwinFat probably leads to estimates different from what would have been obtained from a true random sample of twins, the conclusions on the relative importance of FMI and VO_{2max}/FFM are essentially the same as with GEMINAKAR, which has a more random sampling protocol.

Study III seems to have multiple strengths compared with previous studies. Primarily, the measure used for CRF (VO_{2max}/FFM) is probably not confounded by adiposity, unlike the often-used methods of VO_{2max}/weight or CRF estimated from weight-bearing exercise. Furthermore, studying intrapair differences within MZ pairs allows the estimation of unique environmental

associations, while controlling for the effect of possible confounding by genetic and shared environmental factors. Although the demonstrated strong unique environmental associations between FMI and metabolic health are not direct evidence for adiposity causing metabolic dysfunction, these associations outline opportunities for possible environmental modification of metabolic health through modifying adiposity.

For future research, I suggest that exercise capacity should not be treated as a measure of CRF independent of adiposity, as at least Krachler et al. have previously argued[227]. I stress the importance of scaling VO_{2max} measures by fat-free mass, not total body weight, in order to make comparisons of CRF, as Krachler et al.[216], among others, have suggested. If body weight is used for scaling, it should be exponentiated with an exponent of around 0.67 ($weight^{0.67}$) to reduce bias against subjects with high fat mass[213], although probably a better way to study the effects of CRF apart from adiposity would be to regress the outcome by continuous measures of VO_{2max} and adiposity. Studies should not categorize a CRF measure confounded by adiposity into groups and afterwards try to control for adiposity because this inevitably leads to residual confounding[205]; to avoid this problem, continuous measures should be used when controlling for adiposity[302].

To summarize, the results from Study III show significant phenotypic and unique environmental associations between FMI and worse metabolic health. Associations were strong for insulin sensitivity or resistance, a continuous metabolic syndrome score, VAT amount, and liver fat % ($|\beta|$ from 0.53 to 0.98). There were significant weak or moderate associations for fasting glucose, blood pressure, LDL cholesterol, HDL cholesterol, and triglycerides ($|\beta|$ from 0.20 to 0.47). For FFMI and VO_{2max}/FFM the associations with these metabolic health variables were generally very weak and mostly non-significant ($|\beta|$ from 0.00 to 0.18) both in individuals and intrapair. Our results underscore the importance of adiposity for metabolic health and support the notion that interventions or long-term environmental changes targeted to reduce adiposity would improve metabolic health. Our results cast doubt on the utility of interventions targeting VO_{2max} specifically and the relevance of VO_{2max} for metabolic health more generally. This does not mean that exercise and physical activity are not important, as their beneficial effects might not be mediated through VO_{2max}.

6.6 METHODOLOGICAL CONSIDERATIONS

The interpretation of the demonstrated associations between MZ twin intrapair differences warrants some discussion. I do not know of any formal statistical work demonstrating that the associations between MZ twin intrapair differences ($r_{MZ}(\Delta X, \Delta Y)$) gives similar estimates as the unique environmental correlations (r_E) estimated with classical Cholesky decomposition models on MZ and DZ twins. Nevertheless, I interpret the

$r_{MZ}(\Delta X, \Delta Y)$ (and multiple regression models on intrapair differences in Study III) similarly to r_E; I assume that they both estimate the unique environmental associations between X and Y. Comparing the results from these two different methods in the dataset of Study III show that they give remarkably similar estimates (unpublished results, not shown), with estimates of correlations $r_{MZ}(\Delta X, \Delta Y)$ and r_E differing by ≤ 0.03. Some minor differences between the methods can arise, but they should roughly yield similar estimates. In theory, as both approaches control for common genetic and shared environmental factors, they can be viewed as similar approaches.

Furthermore, a high unique environmental correlation (r_E estimated as the $r_{MZ}(\Delta X, \Delta Y)$ in our studies) between variables does not imply that the unique environmental correlation accounts for a majority of the total phenotypic correlation (r_p), since the unique environmental correlation (r_E or $r_{MZ}(\Delta X, \Delta Y)$) is standardized to unique environmental variance, not to total phenotypic variance. The unique environmental correlation (r_E) can be standardized to total phenotypic variance: $r_e = \sqrt{e_X^2 \times e_Y^2} \times r_E$, where e_X^2 and e_Y^2 stand for the proportion of phenotypic variance in X or Y accounted for by unique environmental variance in X or Y (which are analogous to heritability h_X^2, but related to the unique environmental factors). But since in an ACE model the upper limit of e_x^2 is $1 - h_x^2$ (one minus the heritability of X), traits with high heritability (e.g. obesity and T2DM) will invariably have rather low unique environmental correlations when the correlations are scaled to total phenotypic variance (r_e), even if they have high unique environmental correlations scaled to unique environmental variance (r_E). This is because heritable variation accounts for a significant portion of the phenotypic variation, which by definition is excluded when modeling unique environmental associations. A limitation of the $r_{MZ}(\Delta X, \Delta Y)$ method is that it does not allow for the estimation of heritability (h_X^2) or unique environmental variance (e_X^2), which would allow one to estimate the part of the phenotypic correlation that is accounted by unique environmental factors (r_e). By virtue of controlling for genetic and shared environmental effects, their contributions to the phenotypic correlations are lost in the model. A Cholesky decomposition model for a sample of MZ and DZ twins can however provide these estimates and provide a fuller picture.

There are some caveats that need to be considered with respect to the limitations of Studies I, II, and III. None of them were preregistered or had a complete analysis plan before analyzing the data. Some decisions regarding the way data should be analyzed and reported were made after having performed some analyses on the data. A strict interpretation of P-values in a null hypothesis significance testing framework requires that there is no flexibility in data gathering, processing, or analysis that is not included in the calculation of P-values, but these assumptions are rarely fulfilled in biomedical basic research. Of course, I have avoided making decisions regarding data transformation, handling of outliers, and statistical models based on the results (or their statistical significance). Regardless, having had some

flexibility in the data analysis procedures probably resulted in a higher number of false-positive findings than that implied by the P-values. As a related issue, we only reported unadjusted nominal P-values despite all of the studies involving multiple comparisons, so the family-wise error rate (the probability of making one or more false-positive findings) is actually higher than the 5% implied by the nominal significance level of $P < 0.05$. These abovementioned limitations apply to Studies I and II and not so much to Study III since Studies I and II had relatively low sample sizes and more flexibility in the analysis. Furthermore, the results of Study III were interpreted more with respect to effect sizes rather than statistical significance since in a study with a very high power even practically meaningless effects are significant.

7 CONCLUSIONS

Our results from Studies I and II suggest that the expression of the SAT NAD^+/SIRT1 pathway is reduced by acquired obesity and increased during weight loss. In Study I, the reduced expression of *SIRTs* and NAD^+ synthesis genes in the SAT of heavier co-twins of BMI-discordant MZ pairs show that long-term environmental factors leading to obesity affect the expression of these genes. There was a similar trend for increased total PARP activity in SAT of the heavier co-twins, which, combined with the decreased NAD^+ synthesis gene expression, suggests that acquired obesity leads to decreased NAD^+ availability in SAT and subsequent suppression of SIRT activity. Additionally, in Study I we show negative associations of SAT *SIRT1* expression with insulin resistance and SAT inflammation, which suggests that the decreased SAT SIRT1 activity could in part explain the insulin resistance and inflammation observed in obesity.

In Study II, we showed that SAT *SIRT1* expression mirrors the changes in weight during the weight loss intervention; *SIRT1* expression was increased after weight loss, but reverted to baseline levels after weight regain in a subset of the participants. Additionally, we showed that SAT *NAMPT* expression (the rate-limiting step of NAD^+ synthesis through the salvage pathways) was increased by weight loss and that the SAT total PARP activity was reduced after weight loss, which together suggest that weight loss increases the NAD^+ availability in SAT and subsequently might lead to SIRT activation. To conclude, together the results from Studies I and II suggest that the activity of the SAT NAD^+/SIRT1 pathway is responsive to environmental changes leading to obesity or weight loss (e.g. dietary factors and physical activity) and underline the potential importance of maintaining cellular NAD^+ and SIRT levels via a healthy lifestyle. Our results also support the testing and development of pharmacological interventions aimed at increasing NAD^+ levels[170,300] or SIRT1 activity[133] in humans.

Our results in Study III involving Danish and Finnish twins show that in a multivariate model containing measures of adiposity (FMI), fat-free mass (FFMI), and CRF (VO_{2max}/FFM), adiposity is independently, strongly and harmfully associated with some of the studied metabolic health variables (insulin sensitivity, acute insulin response index, a continuous metabolic syndrome score, VAT amount, and liver fat %, $|\beta|$'s ranging from 0.48 to 0.98), even after controlling for the effect of genetic and shared environmental factors on the associations with the MZ twin intrapair differences approach. The associations of adiposity with other metabolic health variables (fasting glucose, blood pressure, LDL, HDL, and triglycerides) were more modest (with $|\beta|$'s ranging from 0.20 to 0.47), but mostly statistically significant. The similar associations of CRF (VO_{2max}/FFM) were very weak and mostly statistically non-significant ($|\beta| \leq 0.16$). This seems to indicate, that CRF (or

more precisely VO_{2max}) is not meaningfully associated with the measured metabolic health variables in the studied population of healthy adult twins. This is in contrast with multiple earlier studies purporting to show stronger associations, but as reviewed earlier this might be due to the various used measures of CRF being confounded by adiposity since estimating CRF from performance in a weight-bearing exercise[227] or scaling VO_{2max} by dividing it with weight[211,216,217] both underestimate CRF or VO_{2max} in more obese individuals. If body composition is not adequately controlled statistically, these CRF measures correlated with adiposity might lead one to conclude that there are strong associations between CRF and metabolic health, even if in reality these associations are explained by adiposity. Our results from Study III do not, however, imply that physical activity is not important for metabolic health because, as reviewed earlier, VO_{2max} is not a direct measure of physical activity and does not straightforwardly correspond to what is perhaps meant by physical fitness in lay terms. To conclude, the results from Study III underscore the importance of lifestyle and other environmental factors that modify adiposity as major determinants of metabolic health.

Although only Studies I and II are directly thematically related to each other because they both investigate the NAD^+/SIRT1 pathway in SAT, and Studies I and III are methodologically similar, all the studies share the same methodological goal: to estimate the effects of unique environmental factors in explaining associations between the studied variables. In Studies I and III, this was achieved through examining MZ twins, which are practically identical with respect to genetic and shared environmental factors. Because in practice any differences between MZ twins must be due to unique environmental effects, associations between intrapair differences in two variables must be due to unique environmental factors. In Study II, the same goal was achieved by evaluating how unrelated individuals react to an environmental intervention (the weight loss intervention), keeping the genetic and earlier environmental factors constant by virtue of the longitudinal study design. Although only Study II enables assessing which environmental factors (the weight loss intervention) affected the studied variables, all of the studies outline the possibilities of environmental modification of these variables by keeping genetic and some environmental factors fixed.

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